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(54) Title: THERAPEUTIC USE OF AN INHIBITOR OF A HEDGEHOG OR A HEDGEHOG-RELATED SIGNALLING PATHWAY

(57) Abstract: Use of an inhibitor of a Hedgehog signalling pathway, or an inhibitor of a pathway which is a target of the Hedgehog signalling pathway in the preparation of a medicament for treatment of epithelial cell hyperplasia, fibrosis of tissue, inflammation, cancer or an immune disorder. Also a transgenic animal or cell line capable of expressing a component or an inhibitor of a hedgehog signalling pathway or a target pathway of the hedgehog signalling pathway.

## Therapeutic Use

### Field of the Invention

- 5 The present invention relates to a novel therapeutic use, a composition for use in such therapy, a method for identifying useful compounds, a vector and a transgenic animal capable of expressing such compounds.

### Background

10

In many tissues, such as the lung and kidney, chronic unresolving inflammation may lead to remodelling in which both epithelial cell hyperplasia and fibrosis occur. In addition, there is an accompanying mononuclear cell infiltration at local sites of inflammation and the induction of immune responses reactive with self antigens. Similar pathology is also observed in chronic rejection of transplanted organs. In general, these diseases are difficult to manage clinically and this is well illustrated by chronic obstructive pulmonary disease where current pharmacological intervention has limited effects. Therefore, the ability to control this dysregulation of epithelial repair processes that drive anti-self immune responses and tissue remodelling will have an important clinical impact.

20

### Summary of the Invention

The formation of epithelial surfaces and the regulation of epithelial cell growth appears to be highly conserved among species. Recent studies in developmental biology have demonstrated the importance of epithelial cell growth factor genes Shh and Wnt 1 and the TGF- $\beta$  superfamily members BMP4 and BMP7. These gene products not only play an important role in developmental epithelial cell growth but also in tissue patterning. The latter requires the coordinated and

25

temporal regulation of cell differentiation programmes. During development cells respond to growth factors which are found within their environment and it is the interpretation of these various growth factor signalling pathways that will determine the differentiation programme that is initiated. To date little is known  
5 of the contribution of these genes in tissue remodelling and fibrosis that are observed in chronic inflammation; although BMP has been implicated in fibrosis in bone disease. We have noted that in diseased lungs there is increased expression of the gene *patched* which is involved in Hedgehog signalling. We have also noted that intratracheal instillation of plasmid DNA for the Hedgehog  
10 gene leads to the development of epithelial cell hyperplasia, fibrosis of tissue and the infiltration of mononuclear cells. The pathology is similar to that observed in interstitial lung disease.

We propose that antagonists of components of Hedgehog signalling and/or  
15 antagonists of components of a signalling pathway which is a target of Hedgehog signalling may prevent and/or reverse diseases such as epithelial cell hyperplasia, tissue fibrosis, chronic inflammation, cancer and also prevent graft rejection.

20 Statements of Invention

In one aspect the present invention provides a method of treatment for epithelial cell hyperplasia, fibrosis of tissue, inflammation, cancer, or an immune disorder comprising the administration of a therapeutically effective amount of an  
25 antagonist of a component of a Hedgehog family member signalling pathway or an antagonist of a component of a signalling pathway which is a target of Hedgehog signalling to an individual in need of the same.

Put another way the present invention provides use of an antagonist of a  
30 component of a Hedgehog family member signalling pathway or an antagonist of

a component of a signalling pathway which is a target of Hedgehog signalling in the preparation of a medicament for the treatment of epithelial cell hyperplasia, fibrosis of tissue, cancer, inflammation, or an immune disorder.

- 5 In one embodiment the Hedgehog family member is Sonic hedgehog, Indian hedgehog or Desert hedgehog.

In one embodiment the pathway which is a target of Hedgehog signalling is a BMP signalling pathway or a Wnt signalling pathway.

10

In one embodiment the antagonist is HIP, cyclopamine, Fzb, Cerberus, WIF-1, Xnr-3, Noggin, Chordin, Gremlin, or Follistatin or a derivative, fragment, variant, mimetic, homologue or analogue thereof.

- 15 In another embodiment the antagonist is an antibody to a component of the Hedgehog signalling pathway or an antibody to a component of the target pathway of Hedgehog signalling.

- 20 In a further embodiment the antagonist is itself a component of the Hedgehog signalling pathway or a component of the target pathway of Hedgehog signalling.

Preferably the method of the present invention relates to the treatment of pulmonary hyperplasia or pulmonary fibrosis.

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- More preferably the method of the present invention relates to the treatment of adult respiratory distress syndrome; chronic obstructive airway disorders including asthma, emphysema and chronic bronchitis; atelectasis; occupational lung disease including silicosis; hypersensitivity diseases of the lung including  
30 hypersensitivity pneumonitis; idiopathic interstitial lung diseases including

idiopathic pulmonary fibrosis, pneumonia including usual interstitial pneumonia, desquamative interstitial pneumonia and acute interstitial pneumonia; and pleural fibrosis.

- 5 In one embodiment the immune disorder is an autoimmune disease or graft rejection.

More particularly, the autoimmune disease may be thyroiditis, insultitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia  
10 gravis, rheumatoid arthritis and lupus erythematosus.

In one embodiment the cancer is an adenocarcinoma.

In another aspect the present invention provides a composition for use in  
15 treatment of epithelial cell hyperplasia, fibrosis of tissue, inflammation, cancer or an immune disorder comprising a therapeutically effective amount of an antagonist of a Hedgehog signalling pathway or an antagonist of the target pathway of Hedgehog signalling and a pharmaceutically acceptable carrier, diluent or excipient.

20

In another aspect the present invention provides a method for identifying a compound that is an inhibitor of a Hedgehog signalling pathway or a target pathway of the Hedgehog signalling pathway comprising the steps of: (a) determining the activity of the signalling pathway in the presence and absence of  
25 said compound; (b) comparing the activities observed in step (a); and (c) identifying said compound as inhibitor by the observed difference in the activity of the pathway in the presence and absence of said compound.

In yet another aspect the present invention provides a vector capable of expressing an antagonist of a component of a Hedgehog signalling pathway or an antagonist of a component of the target pathway of Hedgehog signalling.

- 5 In a further aspect the present invention provides a transgenic animal capable of expressing antagonist of a component of a Hedgehog family member signalling pathway or an antagonist of a component of the target pathway of Hedgehog signalling.
- 10 Various preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:
- Figure 1 shows a schematic representation of HH signalling;  
Figure 2 shows a schematic representation of a component of HH signalling;
- 15 Figure 3 shows a schematic representation of Wnt signalling;  
Figure 4 shows plasmid SPC-SV40;  
Figure 5 shows plasmid SPC-Shh;  
Figure 6 show the results of Example 2;  
Figures 7-9 show the results of Example 3;
- 20 Figure 10 show the results of Example 4;  
Figure 11 show the results of Example 5;  
Figure 12 show the results of Example 6;  
Figure 13 show the results of Example 7;  
Figure 14 show the results of Example 8;
- 25 Figure 15 show the results of Example 9;  
Figure 16 show the results of Example 12; and  
Figure 17 show the results of Example 13.

- For ease of reference a summary of the accompanying sequence listings is given
- 30 below:

- SEQ ID NO:1 shows the deduced amino acid sequence of mouse SHH and SEQ ID NO:2 shows the corresponding nucleic acid sequence;
- SEQ ID NO:3 shows the deduced amino acid sequence of mouse Dvl-1 and SEQ ID NO:4 shows the corresponding nucleic acid sequence;
- 5 SEQ ID NO:5 shows the deduced amino acid sequence of mouse HIP and SEQ ID NO:6 shows the corresponding nucleic acid sequence; and
- SEQ ID NO:7 shows the deduced amino acid sequence of mouse WIF-1 and SEQ ID NO:8 shows the corresponding nucleic acid sequence.
- 10 References and Accession Nos. are herein incorporated by reference.

#### Hedgehog Family Proteins

- All multicellular organisms require cell communication to regulate growth and differentiation in the embryo. One strategy for this is to establish discrete
- 15 organising centres that emit signals to coordinately control cell proliferation and cell fate determination. The *hedgehog* (*hh*) gene was identified originally through the segment polarity phenotype caused by its mutation in *Drosophila*. Genes of the *hh* family have now been isolated from several vertebrate species,
- 20 including mouse, chicken, zebrafish, rat, *Xenopus* and human. The genes not only seem to show a high degree of structural homology both within and between species, but in addition exhibit some remarkable similarities in the ways in which they function in various embryonic processes. In vertebrates, *Sonic hedgehog* (*Shh*) is a key signal in several signalling centres. There are two other
- 25 mammalian HH members, *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*).

A summary of various *hedgehog* genes is given in the following Table 1:

Table 1

Gene	Species
<i>hedgehog</i> ( <i>hh</i> )	<i>Drosophila</i>



<i>Sonic hedgehog (Shh)</i>	Mouse, Human, Rat, <i>Xenopus</i> , Chicken, Zebrafish
<i>Indian hedgehog (Ihh)</i>	Mouse, Human, Chicken
<i>Desert hedgehog (Dhh)</i>	Mouse
<i>Banded hedgehog (X-bhh)</i>	<i>Xenopus</i>
<i>Cepalic hedgehog (X-chh)</i>	<i>Xenopus</i>
<i>tiggy-winkle hedgehog (twhh)</i>	Zebrafish
<i>echidna hedgehog (ehh)</i>	Zebrafish

The classification of genes from different species is based on the comparison of the expression pattern and the amino acid sequence. Of all vertebrate proteins, DHH is most similar to *Drosophila* HH (51% identity over entire length of processed proteins). Amino acid identity among SHH is 93% between mouse and human, 84% between mouse and chicken, 78% between mouse and *Xenopus*, and 68% between mouse and zebrafish. Intraspecies comparison within the mouse reveals 58-63% identity in pairwise combination between SHH, IHH and DHH. Interspecies comparison between the mouse and *Xenopus* reveals highest identities between IHH and XBHH (70%) and DHH and XCHH (64%).

The various Hedgehog proteins consist of a signal peptide, with a highly conserved N-terminal region and a more divergent C-terminal domain. It is understood that the biologically active Hedgehog peptides are formed from a larger precursor protein. In addition to signal sequence cleavage in the secretory pathway, Hedgehog precursor proteins undergo an internal autoproteolytic cleavage. This autocleavage generates an N-terminal peptide (about 19kDa) and a C-terminal peptide (of about 26-28kDa). It is this N-terminal peptide that is necessary for short- and long-range Hedgehog signalling activities in *Drosophila* and vertebrates. The N-terminal peptide stays tightly associated with the surface of cells in which it is synthesised, while the C-terminal peptide is freely diffusable.

### Signalling Pathway

Figure 1 shows one representation of a Hedgehog signalling pathway, with  
5 particular reference to signalling in vertebrates.

Epithelial cells may express the homeodomain transcription factor engrailed (*en*)  
and secrete Hedgehog protein shown for illustrative purposes in the Figure as  
Shh. We have observed that En plays an important role in the maintenance of  
10 lymphocyte survival in the peripheral immune system.

In target cells, HH signalling is mediated by two transmembrane proteins  
patched (Ptc) which has structural similarities to channel and transporter  
proteins, and Smoothened (Smo), a seven-transmembrane protein similar to G-  
15 protein coupled receptors and the Wingless receptor Frizzled (described below).  
Smo is a constitutive activator of HH target genes. Its activity is normally  
repressed by Ptc, and this repression is relieved by HH binding to Ptc. Thus,  
binding of HH to Ptc allows signal transduction leading to activation of the  
transcription factor Gli, which is located in the nucleus of the target cells.

20 The signal reaches Gli through the cytoplasmic complex formed between (1) the  
serine/threonine kinase Fused (Fu), (2) Suppressor of Fused (SU(Fu)); and (3)  
Costal2 (Cos2). Signalling through this complex may be inhibited by the cAMP-  
dependent protein kinase A (PKA) (see Figure 2).

25 Gli acts on target genes wingless (Wnt) and the BMP /activin growth factors.  
Both Wnt and BMP are secreted to the extracellular fluid to bind to their  
receptors. This process is illustrated schematically in Figure 1.

A summary and comparison of components of the Hedgehog signalling pathway is given below in Table 2:

Table 2

<i>Drosophila</i>	Vertebrate
En	En 1,2
hh	Ihh, Dhh, Shh
Ptc	Ptc 1,2
Smo	Smo
Ci	Gli 1-3
Target genes	
Wg	Wnt ~15
Dpp $\equiv$ TGF $\beta$	BMP 8-10

5

The nomenclature may be used interchangeably herein.

Further information on Hedgehog signalling may be found in the following articles: Ingham; Chuang and McMahon; Picicelli *et al*; and Hammerschmidt *et al*.

10

#### BMP signalling pathway

15

Bone morphogenic proteins (BMPs) are multifunctional cytokines, which are members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily. They regulate cellular proliferation, differentiation, apoptosis of various cells types. Activities of BMPs are extracellularly regulated by BMP-binding proteins, Noggin, Chordin, Gremlin Cerberus and Xnr-3. BMPs have been found to block neurogenesis in early development, but this can be resolved by soluble factors, e.g. Noggin and Chordin, which are secreted extracellularly and which bind and

20

neutralize the BMPs preventing them from activating their receptors. In addition, signalling through BMP receptors can inhibit expression of the Notch ligand Delta, which we have previously shown to be important in regulating peripheral immune responses. The Notch signalling pathway is discussed in our  
5 International patent publication No. WO98/20142.

BMPs bind to two different types of serine-threonine kinase receptors, type I and type II. As for type I receptors, BMPs bind to BMP type IA receptor, BMP type IB receptor and activin type I receptor. As for type II receptors, BMPs bind to  
10 BMP type II receptor, activin type II receptor and activin type IIB receptor. In the receptor-ligand complexes, type II receptors phosphorylate type I receptors in the GS domain (rich in glycine, serine and threonine residues) to activate the latter. The activated type I receptors phosphorylate Smad family, which transduces the signals from cytoplasm into nuclei. Smad1, Smad5 and possibly  
15 MADH6 are activated by BMP receptors, form heteromeric complexes with Smad4, and translocate into the nucleus where they may activate transcription of various genes. Smad6 and Smad7 are inhibitory Smads, and may act as autocrine switch-off signals. BMP induced Smad signalling down regulates achaete/scute gene expression which is required for expression of the Notch ligand Delta. As  
20 indicated in Table 2 above, in *Drosophila*, Decapentaplegic (Dpp) is a homolog of mammalian BMPs.

#### Wingless/Wnt signalling pathway

25 We have examined the role for dysregulation of the Wnt signalling pathway in interstitial lung disease. The Wnt genes are targets of the HH pathway, and the Wnt proteins are secreted growth factors which are involved in the regulation of epithelial cell proliferation and differentiation in the lung during embryonic development. We propose that Wnt signalling may also be upregulated during  
30 processes of epithelial cell repair in the lung.

Dishevelled-1 (Dvl-1) is the murine homolog of the fly Dsh gene and functions to transmit signals from the Wnt receptor, Frizzled, to the cytoplasm, where it regulates the kinase activity of a well known serine/threonine kinase, GSK-3b.

5 Over expression of Dsh in fly epithelia leads to oncogenic activation of the epithelium by increasing Wnt signalling.

A representation of this pathway is shown in Figure 3. Wingless (Wg), in *Drosophila*, and, its vertebrate homolog, Wnt signalling pathways regulate cell

10 proliferation. Wg and Wnt are secreted growth factors which are involved in triggering cellular decisions. The Wg/Wnt ligand binds to Frizzled (Fz) family receptor molecules to initiate a signal transduction cascade involving the cytoplasmic protein Dishevelled (Dvl) (Sussman DJ *et al*). The GenBank accession number for *Dvl-1* cDNA is U10115. The complex illustrated in Figure

15 3 is present in the cytoplasm of the target cell. Generally APC blocks signalling; however, in the presence of signalling from Wnt,  $\beta$ -catenin is released and interacts with two transcription factors - Lef-1/TCF-1 resulting in target gene expression. Target genes of Wnt include En and therefore indirectly HH, c-myc and cyclin D1. It will be appreciated that Notch signalling is also regulated by

20 the Wnt pathway, as Dvl has been found to inhibit Notch signalling.

### Inhibitors

The present invention relates to the use of compounds which inhibit or block

25 (antagonise) Hedgehog signalling. Such compounds may be seen as having the effect of downregulating the expression of Hedgehog. Similarly the present invention also relates to the use of compounds which inhibit or block (antagonise) a signalling pathway which is a target of the Hedgehog signalling pathway. Conveniently such compounds may be referred to herein as inhibitors

30 or antagonists.

The invention contemplates that mutations that result in loss of normal function of the regulators of the Hedgehog signalling pathway or regulators of a pathway which is a target of the Hedgehog signalling pathway in human disease states in which lymphocyte infiltration or failure of a cell cycle checkpoint is involved. Gene therapy to restore such regulatory activity would thus be indicated in treating those disease states. Alternatively, it is contemplated that preventing the expression of or inhibiting the activity of such signalling pathways will be useful in treating the disease states. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate such signalling pathways.

Antagonists for each component of the signalling pathway have been identified. These may be summarised as follows in Table 3:

Table 3

Component	Antagonist
HH	Hip (Chuang and McMahon), Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis (Cooper <i>et al</i> ) e.g. cyclopamine (Coventry <i>et al</i> ).
Wnt	Fzb (Leyns <i>et al</i> ), Cerberus (Bouwmeester <i>et al</i> ), Gremlin (Hsu <i>et al</i> ), WIF-1 (Hsieh <i>et al</i> )
BMP	Noggin (Valenzuela <i>et al</i> ), Chordin (Sasai <i>et al</i> ), Cerberus, Gremlin, Xnr-3
Activin	Follistatin (Iemura <i>et al</i> )

HIP (for Hedgehog-interacting protein) is a membrane glycoprotein that binds to at least all three mammalian Hedgehog proteins with an affinity comparable to that of Ptc. HIP appears to attenuate Hedgehog signalling as a result of binding to Hedgehog proteins. Such a negative regulatory feedback loop could also serve

to modulate the response to any Hedgehog signal. The GenBank accession number for HIP is AF116865.

5 Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis have been studied for more than 30 years as potent teratogens capable of inducing cyclopia and other birth defects. It has also been shown that these compounds specifically block the Shh signaling pathway (Cooper *et al*). One example of such a veratrum alkaloid is cyclopamine (11-deoxojervine), a steroid isolated from the desert plant *Veratrum californicum* (Coventry *et al*).

10

Fzb (Frezzed) is a secreted antagonist of Wnt signalling. Fzb contains a domain similar to the putative Wnt-binding region of the Frizzled family of transmembrane receptors, but it lacks all the transmembrane domains resulting in a putative secreted Wnt-binding protein. The GenBank accession numbers for 15 the *Xenopus*, mouse and human Fzb cDNA sequences are U68059, U68058 and U68057, respectively.

Cerberus is a secreted protein and it has been found to be an antagonist of the Wnt and BMP signalling pathways. The GenBank accession number for the 20 *Xenopus* Cerberus cDNA is U64831.

WIF-1 (Wnt-inhibitory factor-1) is a secreted protein which binds to Wnt proteins and inhibitors their activities. GenBank accession numbers for WIF-1 are: human, AF122922; mouse, AF122923; *Xenopus*, AF122924; and zebrafish, 25 AF122925.

Noggin and Chordin bind to BMPs thereby preventing activation of their signalling cascade.

Gremlin is a secreted protein and it has been found to be an antagonist of the Wnt and BMP signalling pathways. The GenBank accession numbers for Gremlin cDNA are: *Xenopus*, AF045798; chick, AF045799; human, AF045800; and mouse, AF045801.

5

Xnr-3 has been found to be an antagonist of BMP signalling pathways.

Follistatin has been found to inhibit others aspects of BMP activity as well as acting as an activin-binding protein.

10

It will also be appreciated that the antagonist may itself be a component of the Hedgehog signalling pathway, or a component of the target pathway of the Hedgehog signalling pathway. Examples of such antagonists include the negative regulators of HH signalling: Ptc, Cos2 and PKA.

15

In a particularly preferred embodiment use is made of PKA. PKA has been implicated in the mechanism of Hh signal transduction because it acts to repress Hh target genes in imaginal disc cells that express Ci. Ci action as transcriptional repressor or activator is contingent upon Hedgehog-regulated, PKA-dependent proteolytic processing.

20

Cyclic AMP (cAMP) is a nucleotide that is generated from ATP in response to hormonal stimulation of cell-surface receptors. cAMP acts as a signaling molecule by activating A-kinase; it is hydrolyzed to AMP by phosphodiesterase (PDE). cAMP levels affect cubitus cleavage and TGF- $\beta$  levels. Specifically, when cAMP levels increase, TGF- $\beta$  levels decrease and this will affect fibrosis, for example. In another embodiment of the invention use is made of cAMP modifiers in treatment. Such modifiers include PDE inhibitors, and beta-agonists such as the beta-adrenergic agonist. For example, it has been found that

25



ptc 1 transcription can be induced by agents activating the cAMP signal transduction pathway.

Antisense nucleic acids (preferably 10 to 20 base pair oligonucleotides) capable of specifically binding to expression control sequences or RNA are introduced into cells (*e.g.*, by a viral vector or colloidal dispersion system such as a liposome). The antisense nucleic acid binds to the target sequence in the cell and prevents transcription or translation of the target sequence. Phosphothioate and methylphosphate antisense oligonucleotides are specifically contemplated for therapeutic use by the invention. The antisense oligonucleotides may be further modified by poly-L-lysine, transferrin polylysine, or cholesterol moieties at their 5' end.

Also comprehended by the present invention are antibody products (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and antigen-binding fragments thereof) and other binding proteins (such as those identified in the assays above). Binding proteins can be developed using isolated natural or recombinant enzymes. The binding proteins are useful, in turn, for purifying recombinant and naturally occurring enzymes and identifying cells producing such enzymes. Assays for the detection and quantification of proteins in cells and in fluids may involve a single antibody substance or multiple antibody substances in a "sandwich" assay format to determine cytological analysis of HH protein levels. The binding proteins are also manifestly useful in modulating (*i.e.* blocking, inhibiting, or stimulating) interactions.

Antibodies may be generated by administering polypeptides or epitope-containing fragments to an animal, usually a rabbit, using routine protocols. Examples of such techniques include those in Kohler and Milstein.

More generally, the antagonist may be selected from polypeptides and fragments thereof, linear peptides, cyclic peptides, synthetic and natural compounds including low molecular weight organic or inorganic compounds. The antagonist may be derived from a biological material such as a component of extracellular  
5 matrix.

Polypeptide substances, such as Noggin or Chordin, may be purified from mammalian cells, obtained by recombinant expression in suitable host cells or obtained commercially. Alternatively, nucleic acid constructs encoding the  
10 polypeptides may be introduced by transfection using standard techniques or viral infection/transduction.

Inhibitors for use according to the present invention may be conveniently identified using a convenient screening procedure.

15 One assay for identifying such inhibitors may involve immobilizing a component of the relevant pathway, e.g. HH, or a test protein, detectably labelling the nonimmobilized binding partner, incubating the binding partners together and determining the amount of label bound. Bound label indicates that  
20 the test protein interacts with the component.

Another type of assay for identifying inhibitors involves immobilizing a component of the pathway, e.g. HH, or a fragment thereof on a solid support coated (or impregnated with) a fluorescent agent, labelling a test protein with a compound  
25 capable of exciting the fluorescent agent, contacting the immobilized component with the labelled test protein, detecting light emission by the fluorescent agent, and identifying interacting proteins as test proteins which result in the emission of light by the fluorescent agent. Alternatively, the putative interacting protein may be  
30 immobilized and the component may be labelled in the assay.

Moreover, such assays for identifying inhibitors may involve: transforming or transfecting appropriate host cells with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA-binding domain and an activating domain; expressing in the host cells a first hybrid DNA sequence encoding a first fusion of part or all of a component of the pathway, e.g. HH, Wnt or BMP, and the DNA binding domain or the activating domain of the transcription factor; expressing in the host cells a second hybrid DNA sequence encoding part or all of a protein that interacts with said component and the DNA binding domain or activating domain of the transcription factor which is not incorporated in the first fusion; evaluating the effect of a test compound on the interaction between said component and the interacting protein by detecting binding of the interacting protein to said component in a particular host cell by measuring the production of reporter gene product in the host cell in the presence or absence of the test compound; and identifying modulating compounds as those test compounds altering production of the reported gene product in comparison to production of the reporter gene product in the absence of the modulating compound. Presently preferred for use in the assay are a *lexA* promoter to drive expression of the reporter gene, the *lacZ* reporter gene, a transcription factor comprising the *lexA* DNA binding domain and the GAL4 transactivation domain, and yeast host cells.

In a particular embodiment described in relation to Hedgehog signalling the appropriate host cell is transformed or transfected with a DNA construct comprising a reporter gene under the control of the Ptc promoter; expressing in said cells a DNA sequence encoding Hedgehog; evaluating the effect of a test compound on the interaction between HH and the Ptc promoter in a particular host cell by measuring the production of reporter gene product in the host cell in the absence and presence of the test compound; and identifying inhibitors as those test compounds reducing the production of the reporter gene product in

comparison to production of the reporter gene product in the absence of the test compound.

5 Analogous assays may be used for inhibitors of the target pathways of Hedgehog signalling. For example, for the Wnt signalling pathway, the ability of a compound to modulate the interaction of Wnt and Fz may be determined. For BMP signalling the ability of a compound to modulate the interaction of BMP and its BMP receptor may be determined.

10 Combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as inhibitors in such assays.

15 The present invention also relates to the use of derivatives, variants, fragments, analogues, homologues and mimetics of the inhibitors mentioned above, including those identifiable using the assay procedures.

20 The term "derivative" as used herein in relation to the polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of, or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein etc., possesses the capability to antagonise the action of the signalling pathway.

25 The term "variant" as used herein in relation to the polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of, or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein etc., possesses the capability to antagonise the action of the signalling pathway.

The term "fragment" as used herein in relation to the polypeptides of the present invention includes a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the aforementioned polypeptide and possesses the capability to antagonise the action  
5 of the signalling pathway.

The term "analogue" as used herein in relation to the polypeptides of the present invention includes any peptidomimetic, i.e. a chemical compound that possess the capability to antagonise the action of the signalling pathway in a similar  
10 manner to the parent polypeptide.

The term "homologue" as used herein in relation to the polypeptides of the present invention includes a polypeptide which has the same evolutionary origin as the subject polypeptide providing that it possesses the capability to antagonise  
15 the action of the signalling pathway.

The term "mimetic" as used herein in relation to the inhibitors of the present invention includes a compound which also possesses the capability to antagonise the action of the signalling pathway in a similar manner to the parent compound.  
20

More particularly, the term "homologue" covers identity with respect to structure and/or function providing the expression product of the resultant nucleotide sequence has the inhibitory activity. With respect to sequence identity (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more  
25 preferably at least 90% sequence identity. More preferably there is at least 95%, more preferably at least 98%, sequence identity. These terms also encompass allelic variations of the sequences.

Sequence identity with respect to the sequences can be determined by a simple  
30 "eyeball" comparison (i.e. a strict comparison) of any one or more of the

sequences with another sequence to see if that other sequence has, for example, at least 75% sequence identity to the sequence(s).

Relative sequence identity can also be determined by commercially available  
5 computer programs that can calculate % identity between two or more sequences  
using any suitable algorithm for determining identity, using for example default  
parameters. A typical example of such a computer program is CLUSTAL.  
Advantageously, the BLAST algorithm is employed, with parameters set to  
default values. The BLAST algorithm is described in detail at  
10 [http://www.ncbi.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nih.gov/BLAST/blast_help.html), which is incorporated herein  
by reference. The search parameters are defined as follows, can be  
advantageously set to the defined default parameters.

Advantageously, "substantial identity" when assessed by BLAST equates to  
15 sequences which match with an EXPECT value of at least about 7, preferably at  
least about 9 and most preferably 10 or more. The default threshold for  
EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm  
20 employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these  
programs ascribe significance to their findings using the statistical methods of  
Karlin and Altschul (see [http://www.ncbi.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nih.gov/BLAST/blast_help.html)) with  
a few enhancements. The BLAST programs were tailored for sequence  
similarity searching, for example to identify homologues to a query sequence.  
25 For a discussion of basic issues in similarity searching of sequence databases,  
see Altschul *et al* (1994) Nature Genetics 6:119-129.

The five BLAST programs available at <http://www.ncbi.nlm.nih.gov> perform the  
following tasks:

30

**blastp** - compares an amino acid query sequence against a protein sequence database.

5   **blastn** - compares a nucleotide query sequence against a nucleotide sequence database.

**blastx** - compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

10   **tblastn** - compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

**tblastx** - compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

15   BLAST uses the following search parameters:

HISTOGRAM - Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

20   DESCRIPTIONS - Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page).

25   EXPECT - The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported.

30   Fractional values are acceptable. (See parameter E in the BLAST Manual).

CUTOFF - Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least  
5 as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

10 ALIGNMENTS - Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the  
15 BLAST Manual).

MATRIX - Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The valid alternative choices include: PAM40, PAM120,  
20 PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND - Restrict a TBLASTN search to just the top or bottom strand of the  
25 database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER - Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993)  
30 Computers and Chemistry 17:149-163, or segments consisting of short-



periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see <http://www.ncbi.nlm.nih.gov>). Filtering can eliminate statistically significant but biologically uninteresting  
5 reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the  
10 letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other  
15 programs.

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported  
20 against the unfiltered query sequence should be suspect.

NCBI-gi - Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

25 Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at <http://www.ncbi.nlm.nih.gov/BLAST>.

Other computer program methods to determine identify and similarity between the two sequences include but are not limited to the GCG program package

(Devereux *et al* 1984 Nucleic Acids Research 12: 387) and FASTA (Atschul *et al* 1990 J Molec Biol 403-410).

5 In some aspects of the present invention, no gap penalties are used when determining sequence identity.

The present invention also encompasses use of nucleotide sequences that are complementary to the sequences presented herein, or any fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence  
10 can be used as a probe to identify similar promoter sequences in other organisms.

The present invention also encompasses use of nucleotide sequences that are capable of hybridising to the sequences presented herein, or any fragment or derivative thereof.

15

Hybridization means a "process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY) as well as the process of amplification as carried out in polymerase chain reaction technologies as  
20 described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

Also included within the scope of the present invention are use of nucleotide sequences that are capable of hybridizing to the nucleotide sequences presented  
25 herein under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

30

Maximum stringency typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe); high stringency at about  $5^\circ\text{C}$  to  $10^\circ\text{C}$  below  $T_m$ ; intermediate stringency at about  $10^\circ\text{C}$  to  $20^\circ\text{C}$  below  $T_m$ ; and low stringency at about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related nucleotide sequences.

In a preferred aspect, the present invention covers use of nucleotide sequences that can hybridise to the nucleotide sequences of the present invention under stringent conditions (e.g.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$ ).

The present invention also encompasses use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any fragment or derivative thereof. Likewise, the present invention encompasses use of nucleotide sequences that are complementary to sequences that are capable of hybridising to the sequence of the present invention. These types of nucleotide sequences are examples of variant nucleotide sequences.

In this respect, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences presented herein. Preferably, however, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (eg.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$  { $1\times\text{SSC} = 0.15\text{ M NaCl}$ ,  $0.015\text{ M Na}_3\text{ citrate pH } 7.0$ }) to the nucleotide sequences presented herein.

#### Transgenic Animals

The present invention also relates to transgenic animals which are capable of expressing or overexpressing at least one antagonist useful in the present

invention. Preferably the animal expresses or overexpresses HIP, Fzb-1, Noggin (Ngg) and/or WIF-1.

5 The present invention additionally relates to transgenic animals which are capable of expressing or overexpressing at least one polypeptide which is a component of the Hedgehog signalling pathway or a component of a pathway which is a target of the Hedgehog signalling pathway, such as the Wnt or BMP signalling pathway. Preferably the animal expresses or overexpresses HH (more preferably Shh), and/or Dvl-1.

10

The transgenic animal is typically a vertebrate, more preferably a rodent, such as a rat or a mouse, but also includes other mammals such as human, goat, pig or cow etc.

15 Such transgenic animals are useful as animal models of disease and in screening assays for new useful compounds. By specifically expressing one or more polypeptides, as defined above, the effect of such polypeptides on the development of disease can be studied. Furthermore, therapies including gene therapy and various drugs can be tested on transgenic animals. Methods for the  
20 production of transgenic animals are known in the art. For example, there are several possible routes for the introduction of genes into embryos. These include (i) direct transfection or retroviral infection of embryonic stem cells followed by introduction of these cells into an embryo at the blastocyst stage of development; (ii) retroviral infection of early embryos; and (iii) direct microinjection of DNA  
25 into zygotes or early embryo cells.

The present invention also includes stable cell lines for use as disease models for testing or treatment.

A stable cell line will contain a recombinant gene or genes, also known herein as a transgene, encoding one or more inhibitors or components of a Hedgehog signalling pathway or of a pathway which is a target of the Hedgehog signalling pathway.

5

Preferably the transgene is HH (more preferably Shh), HIP, WIF-1, Fzb-1, Ngg and/or Dvl-1. A cell line containing a transgene, as described herein, is made by introducing the transgene into a selected cell line according to one of several procedures known in the art for introducing a foreign gene into a cell.

10

As also described below, the sequences encoding the inhibitors and components of signalling pathways, as described herein, are operably linked to control sequences, including promoters/enhancers and other expression regulation signals.

15

The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of  $\alpha$ -actin,  $\beta$ -actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). Tissue-specific promoters specific for lymphocytes, dendritic cells, skin, brain cells and epithelial cells within the eye are particularly preferred, for example the CD2, CD11c, keratin 14, Wnt-1 and Rhodopsin promoters respectively. Preferably the epithelial cell promoter SPC is used. They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat

20  
25  
30

(MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

5 It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

10 In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

#### Therapeutic Uses

15 As previously mentioned, in many tissues, such as lung and kidney, inflammation may lead to chronic diseases. For example, chronic lung disease involve tissue remodelling in the presence of inflammatory cells, examples are emphysema and interstitial lung disease (ILD). The disease tissue is associated  
20 with epithelial cell hyperplasia leading to fibrosis and scarring. There is an accompanying mononuclear cell infiltration at local sites of inflammation and the induction of immune responses reactive with self antigens. Similar pathology is also observed in the chronic rejection of graft tissue, including transplanted organs.

25 Hedgehog is important in regulating growth and differentiation of epithelial cells. It has a role in the formation of notochord, limb, gut, lung, skin etc. Branching morphogenesis occurs through induction of Wnt and BMP growth factors. It binds to its receptor Ptc and Smo. Mutations can lead to the human  
30 autosomal disorder Nevroid basal cell carcinoms syndrome (NBCCS) which is

characterised by developmental abnormalities and a high predisposition for various forms of cancer mainly the very common basal cell carcinomas (BCC).

5 BMPs are members of the TGF- $\beta$  superfamily. Whereas a role for TGF- $\beta$ 1 in mediating lung fibrosis is well established, previously BMP-4 has only been implicated in fibrotic disease of bone.

We now provide a method for the treatment of epithelial cell hyperplasia and fibrosis particularly in the lung and kidney. More particularly diseases which  
10 may be treated include adult respiratory distress syndrome; chronic obstructive airway disorders/ chronic obstructive pulmonary disease including asthma, emphysema and chronic bronchitis; atelectasis; occupational lung disease including silicosis; hypersensitivity diseases of the lung including hypersensitivity pneumonitis; idiopathic interstitial lung diseases including  
15 idiopathic pulmonary fibrosis, usual interstitial pneumonia, desquamative interstitial pneumonia and acute interstitial pneumonia; and pleural fibrosis. Further details on such conditions and those given below may be found in The Merck Manual (17th Edition), published by Merck Research Laboratories, N.J., USA.

20 The present invention is also useful in treating immune disorders such as autoimmune diseases or graft rejection such as allograft rejection.

Examples of disorders that may be treated include a group commonly called  
25 autoimmune diseases. The spectrum of autoimmune disorders ranges from organ specific diseases (such as thyroiditis, insulinitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis) to systemic illnesses such as rheumatoid arthritis or lupus erythematosus. Other disorders include immune hyperreactivity, such as allergic reactions.

30

In more detail: Organ-specific autoimmune diseases include multiple sclerosis, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic), autoimmune hepatitis, thyroiditis, insulinitis, iridocyclitis, skleritis, uveitis, orchitis, myasthenia gravis, idiopathic thrombocytopenic purpura,  
5 inflammatory bowel diseases (Crohn's disease, ulcerative colitis).

Systemic autoimmune diseases include: rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, sjogren's syndrom, undifferentiated connective tissue syndrome, antiphospholipid syndrome, different forms of  
10 vasculitis (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essentiell (mixed) cryoglobulinemia, Psoriasis vulgaris and psoriatic arthritis,  
15 diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatoid granulomatosis, erythema nodosum, ankylosing spondylitis, Reiter's syndrome, different forms of inflammatory dermatitis.

20 A more extensive list of disorders includes: unwanted immune reactions and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease,  
25 reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other  
30 renal and urologic diseases, otitis or other oto-rhino-laryngological diseases,



dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fundus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock,

infectious diseases, inflammatory complications or side effects of surgery or organ, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat  
5 or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

10

The present invention is also useful in cancer therapy, particularly in diseases involving the conversion of epithelial cells to cancer. The present invention is especially useful in relation to adenocarcinomas such as: small cell lung cancer, and cancer of the kidney, uterus, prostate, bladder, ovary, colon and breast.

15

We have now found that the use of antagonists of Hedgehog signalling may prevent and/or promote regression of the above-mentioned diseases.

#### Vectors, host cells, expression

20

The present invention also relates to vectors which comprise a polynucleotide useful in the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides useful in the present invention by such techniques.

25

For recombinant production, host cells can be genetically engineered to incorporate expression systems or polynucleotides of the invention. Introduction of a polynucleotide into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis *et al* and Sambrook *et al*, such  
30 as calcium phosphate transfection, DEAE-dextran mediated transfection,

transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. Coli*, streptomyces and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used to produce a polypeptide useful in the present invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

#### Methods of Delivery

In the present invention the polynucleotide may be delivered to a target cell population, either *ex vivo* or *in vivo*, by any suitable Gene Delivery Vehicle.

This includes but is not restricted to, DNA, formulated in lipid or protein complexes or administered as naked DNA via injection or biolistic delivery, viruses such as retroviruses, adenoviruses, herpes viruses, vaccinia viruses, adeno associated viruses. The GDV can be designed by a person ordinarily skilled in the art of recombinant DNA technology and gene expression to express the fusion protein at appropriate levels and with the cellular specificity demanded by a particular application.

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a

unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

The vector can be delivered by viral or non-viral techniques.

5

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

- 10 Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-
- 15 (trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

- Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a
- 20 lentiviral vector or a baculoviral vector.

- Examples of retroviruses include but are not limited to: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV),
- 25 Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

- 5 Adenoviruses and adeno-associated viruses which have good specificity for epithelial cells are particularly preferred.

Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA  
10 biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

Thus, nucleic acid vectors according to the invention may be capable of delivery preferentially to the target cell. For example in the case of a retroviral vector, the retroviral envelope protein may be capable of directing the vector to a  
15 particular cell type or cell types. For that purpose, the envelope protein may be a modified envelope protein adapted to have a specific targeting ability, or it may be a selected envelope protein derived from a different viral or retroviral source and having the desired targeting ability.

20 Preferably, the nucleic acid in a vector according to the invention is operatively linked to an expression control sequence capable of causing preferential expression of the fusion protein in the target cell. The expression control sequence may be for example a promotor or enhancer which is preferentially active in certain cell types including the target cell, or a promotor or enhancer  
25 which is preferentially active under certain conditions.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

- 5 Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of a nucleic acid in one tissue while remaining largely "silent" in other tissue types. A particularly preferred promoter is the epithelial cell promoter.
- 10 The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

#### Administration

- 15 Compounds capable of affecting a component of the Hedgehog family signalling pathway or a target pathway thereof for use in therapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the compound identified and the route of administration but
- 20 typically they can be formulated for topical, parenteral, intramuscular, intravenous, intra-peritoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The compound may be used in an injectable form. It may therefore be mixed with any vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be
- 25 treated, although it may be administered systemically.

- The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The compounds of the present invention may be admixed with any
- 30 suitable binder(s), lubricant(s), suspending agent(s), coating agent(s),

solubilising agent(s). It is also preferred to formulate the compound in an orally active form.

In general, a therapeutically effective daily oral or intravenous dose of the compounds of the invention, including compounds of formula (1) and their salts, is likely to range from 0.01 to 50 mg/kg body weight of the subject to be treated, preferably 0.1 to 20 mg/kg. The compounds of the formula (I) and their salts may also be administered by intravenous infusion, at a dose which is likely to range from 0.001-10 mg/kg/hr.

Tablets or capsules of the compounds may be administered singly or two or more at a time, as appropriate. It is also possible to administer the compounds in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the compounds of the invention can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.



For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients. or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents.

5

The compositions (as well as the compounds alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent.

10

For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

15 For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For oral, parenteral, buccal and sublingual administration to subjects (such as patients), the daily dosage level of the compounds of the present invention and their pharmaceutically acceptable salts and solvates may typically be from 10 to 500 mg (in single or divided doses). Thus, and by way of example, tablets or capsules may contain from 5 to 100 mg of active compound for administration singly, or two or more at a time, as appropriate. As indicated above, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. It is to be noted that whilst the above-mentioned dosages are exemplary of the average case there can, of course, be individual instances where higher or lower dosage ranges are merited and such dose ranges are within the scope of this invention.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example,  
5 the age, weight and condition of the patient.

The term treatment or therapy as used herein should be taken to encompass diagnostic and prophylactic applications.

10 The treatment of the present invention includes both human and veterinary applications.

As used herein the terms protein and polypeptide and peptide may be assumed to be synonymous, protein merely being used in a general sense to indicate a  
15 relatively longer amino acid sequence than that present in a polypeptide, and polypeptide merely being used in a general sense to indicate a relatively longer amino acid sequence than that present in a peptide. Generally for ease of reference only we will simply refer to the term polypeptide.

20 The invention will now be described in further detail with reference to the following non-limiting examples:

#### **Example 1 : Construction of SPC-Shh expression plasmid**

25 The SPC-mouse Shh vector shown in Figure 5 was constructed from a parent expression vector that was made as follows. The parent vector shown in Figure 4 contains a pUC18 backbone with an ampicillin resistance gene, a 3.7 kb sequence containing the human SPC promoter region, a multiple cloning site (MCS), an SV40 small T intron and a 0.4 kb sequence containing a poly(A)  
30 addition site and with stop codons in all three reading frame (Korfhagen *et al*;

Development 1997) .The cDNA sequence encoding the mouse Shh was cloned into the MCS.

Additional vectors including SPC-HIP, SPC-WIF-1, SPC-Dvl-1 were made by  
5 cloning murine cDNAs for HIP, WIF-1, Dvl-1 into the MCS of the SPC expression vector.

**Example 2 : Increased expression of Ptc in the lung epithelial cells from human patients with idiopathic fibrosing alveolitis (IFA also known as  
10 CFA) and in a murine model of interstitial pulmonary fibrosis (IPF).**

Fig. 6C

BALB/c mice were treated intratracheally with 50 µg of FITC dissolved in physiological buffered saline (PBS). Three months later mice were sacrificed  
15 and lungs removed and fixed in 4% buffered formalin and embedded in paraffin. 5 µm sections of lung tissue were placed onto TESPA coated slides and the expression of Ptc gene expression was examined by anti-sense RNA in situ hybridization (ISH).

20 Sections were hybridized with digoxigenin antisense RNA probes specific for murine Ptc1 at 65°C . The bound probe was detected by alkaline phosphatase conjugated goat anti-digoxigenin Fab and sections were developed using NBT and BCIP as the substrate. We observed increased expression of Ptc in lung epithelial cells in the murine model of IPF. Expression of Ptc was restricted only  
25 to those areas showing damage. Increased Ptc expression was noted within 24 hours of i.t. FITC and was maintained for at least 6 months.

Fig. 6 A & B

Paraffin embedded archive lung tissue from human patients diagnosed with IFA or control patients with healthy lung tissue were sectioned at 5 µm and placed onto TESPA coated slides. The slides were then analysed for Ptc gene expression by anti-sense RNA in situ hybridization (see above). We observed  
5 increased expression of Ptc in lung epithelial cells.

**Example 3 : Overexpression of Shh leads to epithelial cell hyperplasia and lung fibrosis. Figures 7-9**

10 BALB/c mice were injected i.t. with either (i) saline alone, (ii) 20 µg of SPC plasmid dissolved in saline, or (iii) 20 µg of SPC-Shh plasmid DNA dissolved in saline on day 0 and day 5. The SPC plasmid provides tissue-specific expression of a desired gene as it contains the promoter sequence from the lung epithelial cell-specific surfactant protein C (i.e. SPC). Mice were sacrificed at day 12 and  
15 day 35 where upon the lungs were removed and placed into 4% buffered formalin.

5 µm sections of lung tissue (Fig 7, 8) or trachea (Fig. 9) from each group at each of the two time points were placed onto poly-L-Lysine coated slides and  
20 stained using the haematoxylin and eosin (H & E) histochemical stain.

The groups contained:

Day 12 PBS (2 mice), SPC (2 mice) and SPC-shh (2 mice)

Day 35 PBS (3 mice) SPC (3 mice) and SPC-shh (3 mice)

25 Slides from the day 35 treatment group were further analysed for collagen production using a Masons-trichrome histochemical stain. This type of stain is routinely used to stain for collagen fibres in tissue samples which turn green. Increased levels of collagen staining could be identified in lung tissue from SPC-Shh treated mice when compared to controls.

30

- Slides from the day 35 treatment group were also analysed for potential goblet cell hyperplasia using the periodic acid-schiff (PAS) histochemical stain. Using this stain, cells which generate mucins stain an intense pink colour while epithelium stains light blue. We observed an increase in the number of goblet cells in the lung tissue of SPC-Shh mice (i.e. pink cells) and increased mucous secretion into the airways. The increased goblet cell numbers cells were observed only in those airways showing signs of epithelia hyperplasia. Normal airways in the SPC-Shh mice were equivalent to those in the control mice.
- Slides from Day 12 and Day 35 treatment groups were stained for a proliferation index marker, Ki-67, to provide evidence that the lung epithelia of SPC-shh mice were actively proliferating. Slides from the three treatment groups were stained with an antibody specific for the Ki-67 antigen which marks cells in the S-phase of the cell cycle. There was an increase in the number of Ki-67 +ve cells in the lung epithelium of SPC-Shh treated mice when compared to the epithelium of control mice

**Example 4 : Epithelial cells express high levels of Shh following FITC damage**

- Mice were treated intratracheally with the hapten fluorescein isothiocyanate. Seven days later the lungs were removed and fixed in formalin. Sections were cut and stained for Shh by immunohistochemistry. Figs. 10A and 10B show expression of Shh in the lung of FITC treated mice, while Fig. 10C shows the staining for Shh observed in the control lung. Shh could be detected on epithelial cells, and a higher level of Shh was detected on a basal cell population in the lung interstitium consistent in morphology with fibroblasts.

**Example 5 : Epithelial cells express high levels of Shh following FITC damage**

5 Mice were treated intratracheally with the hapten fluorescein isothiocyanate. One month later the lungs were removed and fixed in formalin. Sections were cut and stained for Shh by immunohistochemistry. Figs. 11A and 11B show expression of Shh in two different sections of lung of FITC treated mice, while Figs. 11C and 11D show the staining for Shh observed in the control lung. Shh  
10 could be detected at a higher level on epithelial cells than on a basal cell population in the lung interstitium.

**Example 6 : Epithelial cells express high levels of Ptc following FITC damage**

15 Mice were treated intratracheally with the hapten fluorescein isothiocyanate. Seven days later the lungs were removed and fixed in formalin. Sections were cut and stained for Shh by immunohistochemistry. Fig. 12A shows expression of Ptc in the lung of FITC treated mice, while Fig. 12B shows the staining for Ptc  
20 observed in the control lung. Ptc could be detected on epithelial cells and a higher level of Ptc was detected on infiltrating leukocytes found in the lung interstitium.

**Example 7 : Shh and Ptc staining on biopsy material from human CFA lung**

25 Archive material from a CFA patient was sectioned and stained by immunohistochemistry for the presence of Shh-N (the bioactive protein) and Ptc. Figs. 13A-C show staining for Shh and Figs. 13D-F show Ptc expression. Each Fig. represents a serial section taken from the same piece of lung at 10x. The

cells within the airway interstitium and aveolar space contain leukocytes and these stain strongly for Ptc.

**Example 8 : Shh and Ptc staining on biopsy material from human CFA lung**

5

Archive material from a CFA patient was sectioned and stained by immunohistochemistry for the presence of Shh-N (the bioactive protein) and Ptc. Figs. 14A and B show staining for Shh and Figs. 14C and D show Ptc expression. Figs. 14A and C represents a serial section taken from the same  
10 piece of lung at 10x, while Figs. 14B and D are 40x views taken from the lower portion of the section. The cells within the airway interstitium and aveolar space contain leukocytes and these stain strongly for Ptc.

**Example 9 : Shh and Ptc staining on biopsy material from human CFA lung**

15

Archive material from a CFA patient was sectioned and stained by immunohistochemistry for the presence of Shh-N (the bioactive protein) and Ptc. Figs. 15A and B show staining for Shh and Figs. 15C and D show Ptc expression. Figs. 15A and C represent serial sections taken from the same piece  
20 of lung, while Figs. 15B and D are serial sections taken from another section. The cells within the airway lumen contain aveolar macrophages and these stain strongly for Ptc.

**Example 10 : Effect of introduction of SPC-HIP**

25

Our previous studies have revealed that dysregulation of the Shh signalling pathway during epithelial cell repair in the lung, can lead to lymphocyte infiltration with concomitant induction of interstitial fibrosis and scarring. We have previously used a novel model of pulmonary fibrosis where naive BALB/c  
30 mice were treated i.t. with 50 µg of FITC dissolved in saline (PBS) leads to an

initial strong inflammatory response which resolves by day 7, but EC hyperplasia is evident at this time and lymphocytes begin to infiltrate the lung at the sites of EC hyperplasia by day 21. By day 28 there is evidence of interstitial fibrosis which seems to be aggravated by the presence of lymphocytes in the lung. In this model we have observed increased expression of Ptc-1 in sites of EC hyperplasia but not in normal areas of lung tissue. This indicates that there is dysregulation of the Shh pathway in the disease process in the FITC treated mice.

10 Since HIP is a natural antagonist of the Shh protein, we overexpress HIP in the lung using the SPC expression vector as follows:

BALB/c mice are treated i.t. with 50 µg of FITC dissolved in saline (PBS) and 1-3 months later mice, which are time points where it is known that mice would normally have mild to severe fibrosis respectively, they are given two injections of either SPC plasmid alone in saline or SPC-HIP in saline i.t. 7 days apart. Mice are sacrificed at day 7, day 30 and day 60 post SPC-HIP administration. The lung tissue is removed and fixed in 4% buffered formalin. Sections are examined by H & E, PAS, Masons-trichrome and Ki-67 at the various time points. In addition, we examine the expression of Ptc and Shh by ISH and immunohistochemistry. A reduction of Ptc expression and EC hyperplasia and lung fibrosis is seen when compared to the epithelium of control mice.

#### **Example 11 : Effect of introduction of PKA**

25 BALB/c mice are treated i.t. with 50 µg of FITC dissolved in saline (PBS) and 1-3 months later mice, which are time points where it is known that mice would normally have mild to severe fibrosis respectively, they are given two injections of PKA in saline i.t. 7 days apart. Mice are sacrificed at day 7, day 30 and day 60 post SPC-HIP administration. The lung tissue is removed and fixed in 4%



buffered formalin. Sections are examined by H & E, PAS, Masons-trichrome and Ki-67 at the various time points. In addition, we examine the expression of Ptc and Shh by ISH and immunohistochemistry. A reduction of Ptc expression and EC hyperplasia and lung fibrosis is seen when compared to the epithelium of control mice.

**Example 12 : Epithelial cells express high level of Dvl-1 following FITC damage**

Mice were treated intratracheally with the hapten fluorescein isothiocyanate. Seven days later the lungs were removed and fixed in formalin. Sections were cut and stained for Dvl-1 by immunohistochemistry. Fig. 16A shows expression of Dvl-1 in the of lung of FITC treated mice, while Fig. 16B shows the staining for Dvl-1 observed in the control lung. Strong expression of Dvl-1 on epithelial cells and infiltrating leukocytes was observed.

**Example 13 : Dvl-1 adenovirus induces epithelial cell proliferation**

Mice were treated intratracheally with a control adenovirus (Figs. 17A and C) or an adenovirus containing the murine Dvl-1 cDNA (Figs. 17B and D). Four days later the lungs were removed and fixed in formalin. Sections were cut and stained for the proliferation marker Ki67 by immunohistochemistry. Figs. 17A and B show a view of the lung at 10x and Figs. 17C and D are 40x views of the sections shown in the dotted box. Proliferating cells express the Ki67 antigen at high levels.

**Example 14 : A role for dysregulation of Wnt signalling in lung fibrosis**

The Dvl-1 protein is overexpressed in the lung epithelia of mice to examine what effect it may have on the development of lung fibrosis.

BALB/c mice were injected i.t. with either (i) 20 µg of SPC plasmid dissolved in saline, or (ii) 20 µg of SPC-Dvl-1 plasmid DNA dissolved in saline on day 0 and day 7. Mice were sacrificed at day 14 and day 35 where upon the lungs were removed and placed into 4% buffered formalin.

5

The lung tissue is removed and fixed in 4% buffered formalin. Sections are examined by H & E, PAS, Masons-trichrome and Ki-67 at the various time points. In addition, the expression of Dvl-1, Ptc and Shh is examined by ISH and immunohistochemistry. It was found that overexpression leads to EC

10 hyperplasia and lung fibrosis.

**Example 15 : To examine the effect of introduction of the Wnt antagonist WIF-1**

5 WIF-1 is a novel protein that was recently identified to be expressed as a transmembrane protein which binds to Wnt proteins to neutralize them. WIF-1 is normally expressed in the lung tissue, as well as the brain and so we performed a similar series of experiments as for the SPC-HIP protocols using SPC-WIF-1 in its place. Again a reduction in EC hyperplasia and lung fibrosis is seen when compared to the epithelium of control mice.

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Claims

1. Use of an inhibitor of a Hedgehog signalling pathway, or an inhibitor of a  
5 pathway which is a target of the Hedgehog signalling pathway in the preparation  
of a medicament for treatment of epithelial cell hyperplasia, fibrosis of tissue,  
inflammation, cancer or an immune disorder.
2. Use of claim 1 wherein the Hedgehog signalling pathway is the Sonic  
10 hedgehog, Indian hedgehog or Desert hedgehog signalling pathway.
3. Use of claim 1 wherein the pathway which is a target of the Hedgehog  
signalling pathway is the Wnt or BMP signalling pathway.
- 15 4. Use of any preceding claim in which the inhibitor is HIP, cyclopamine,  
Fzb, Cerberus, WIF-1, Xnr-3, Noggin, Chordin, Gremlin, or Follistatin or a  
derivative, fragment, variant, mimetic, homologue or analogue thereof.
5. Use of any one of claims 1 to 4 in which the inhibitor is Ptc, Cos2 or  
20 PKA or an agent of the cAMP signal transduction pathway.
6. Use of any one of claims 1 to 4 wherein the inhibitor is an antibody.
7. Use of any preceding claim for the preparation of a medicament for the  
25 treatment of the lung or kidney.
8. Use of any preceding claim for the preparation of a medicament for the  
treatment of adult respiratory distress syndrome; chronic obstructive airway  
disorders including asthma, emphysema and chronic bronchitis; atelectasis;

occupational lung disease including silicosis; hypersensitivity diseases of the lung including .

hypersensitivity pneumonitis; idiopathic interstitial lung diseases including  
5 idiopathic pulmonary fibrosis, pneumonia including usual interstitial pneumonia, desquamative interstitial pneumonia and acute interstitial pneumonia; and pleural fibrosis.

9. Use of any preceding claim in which the immune disorder is an  
10 autoimmune disease or graft rejection.

10. Use according to claim 9 in which the autoimmune disease is thyroiditis, insulinitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis, rheumatoid arthritis and lupus erythematosus.

15

11. Use according to any preceding claim in which the cancer is an adenocarcinoma.

12. A composition for use in treatment of epithelial cell hyperplasia, fibrosis  
20 of tissue, inflammation, cancer or an immune disorder comprising a therapeutically effective amount of an inhibitor of a Hedgehog signalling pathway or an inhibitor of a target pathway of the Hedgehog signalling pathway and a pharmaceutically acceptable carrier, diluent or excipient.

25 13. A method for identifying a compound that is an inhibitor of a Hedgehog signalling pathway or a target pathway of the Hedgehog signalling pathway comprising the steps of: (a) determining the activity of the signalling pathway in the presence and absence of said compound; (b) comparing the activities observed in step (a); and (c) identifying said compound as inhibitor by the

observed difference in the activity of the pathway in the presence and absence of said compound.

14. Use of an inhibitor identifiable using the method of claim 13 in a use of  
5 any one of claims 1 to 11 or a composition of claim 12.

15. A vector capable of expressing an inhibitor of a Hedgehog signalling pathway or a target pathway of the Hedgehog signalling pathway.

10 16. A transgenic animal or cell line capable of expressing an inhibitor of a Hedgehog signalling pathway or a target pathway of the Hedgehog signalling pathway.

15 17. A transgenic animal or cell line according to claim 16 wherein the inhibitor is WIF-1, Fzb-1, Noggin or HIP.

18. A transgenic animal or cell line capable of expressing a component of the Hedgehog signalling pathway or a component of pathway which is a target of the Hedgehog signalling pathway.  
20

19. A transgenic animal or cell line according to claim 18 wherein the component is Sonic Hedgehog.

20. Use of a transgenic animal or cell line according to any of claims 16 to  
25 19 as a disease model or in a method according to claim 13.

1 / 15

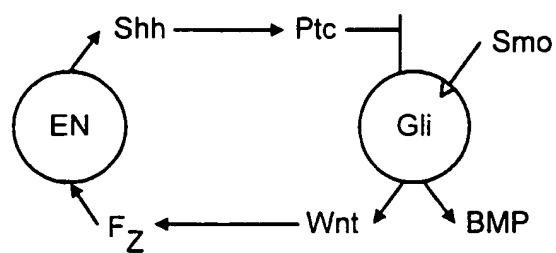


FIG. 1

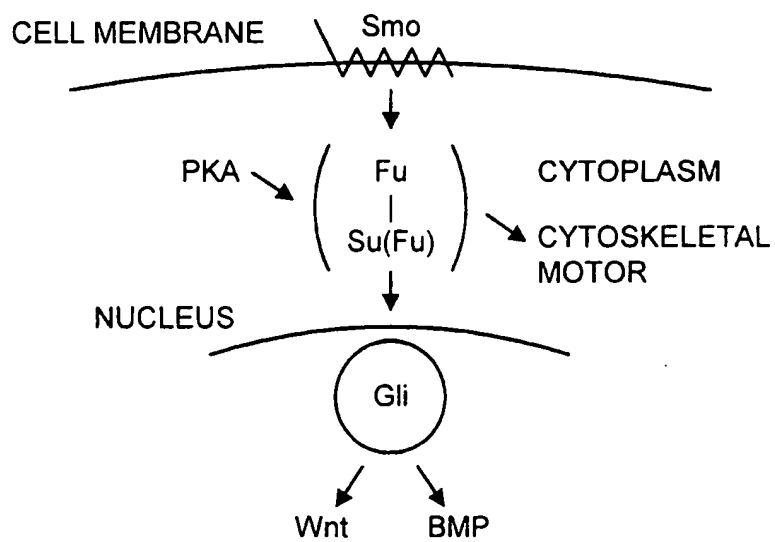


FIG. 2



2 / 15

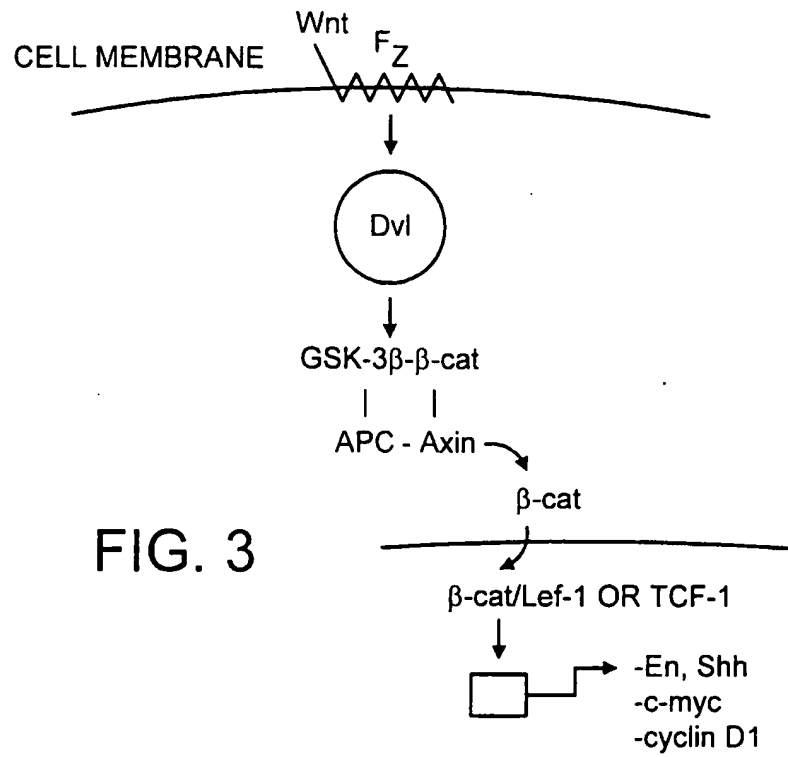
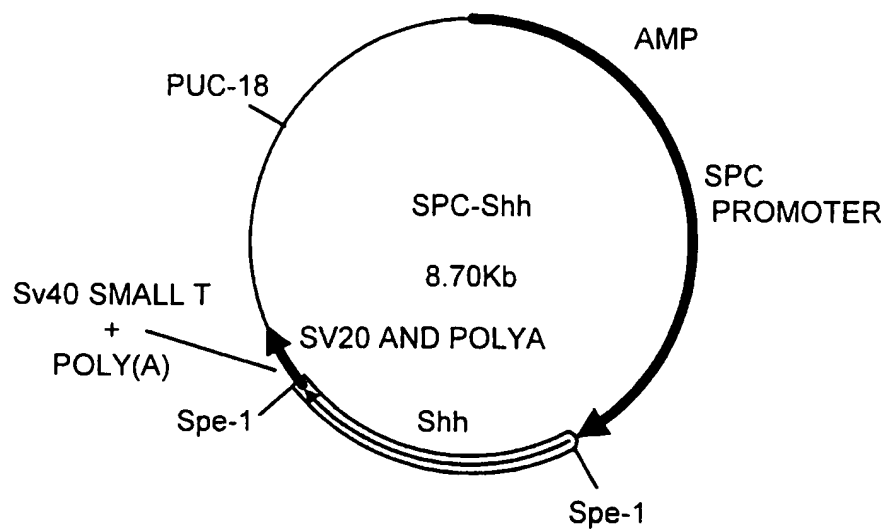
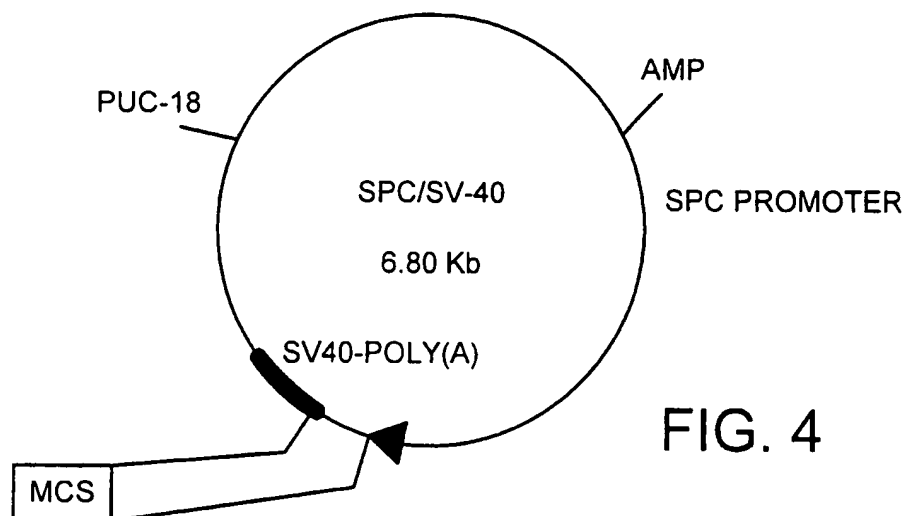


FIG. 3

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**FIG. 5**

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## FIG. 6

PTC *in situ* HYBRIDISATION



a) NORMAL HUMAN LUNG

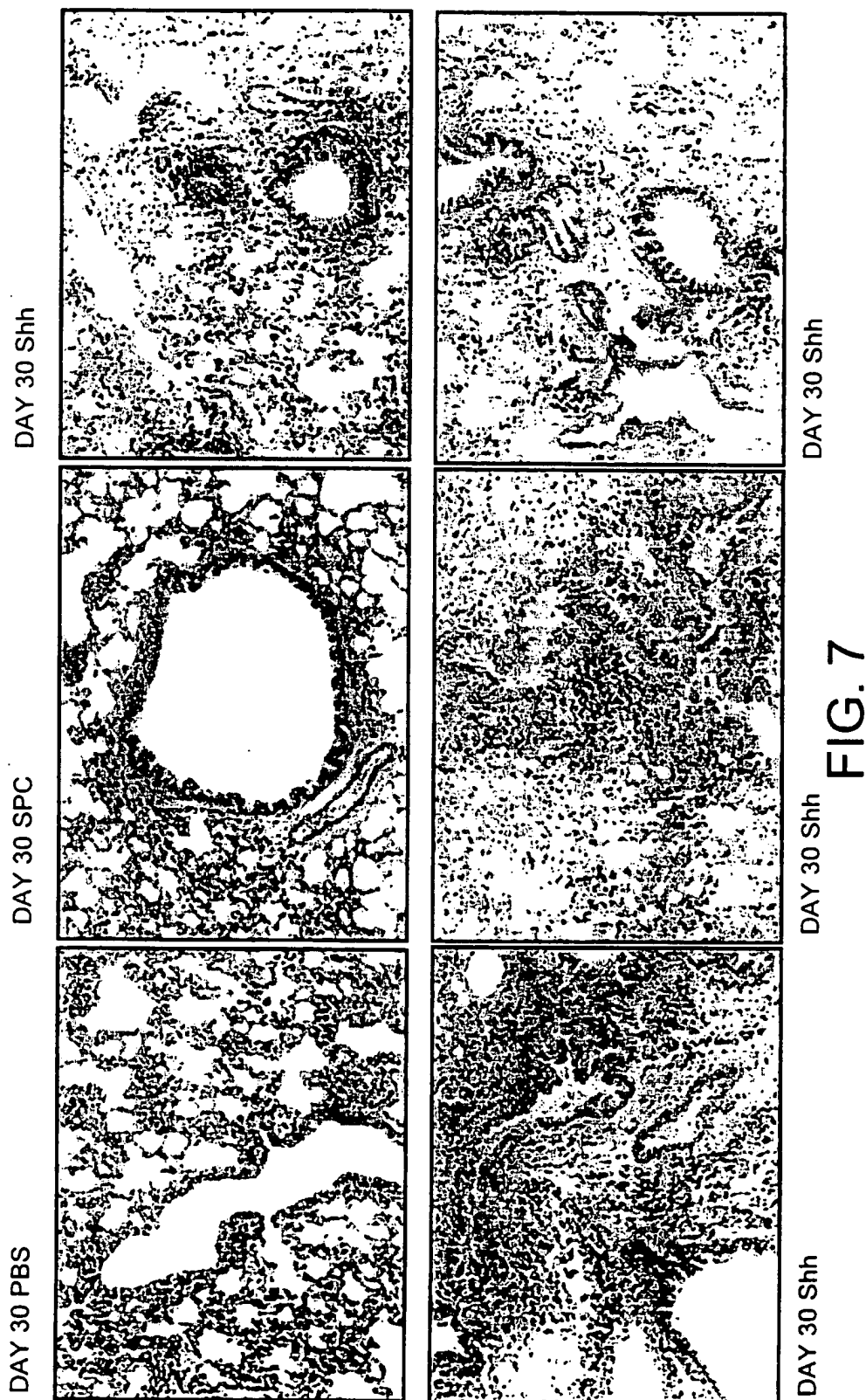


b) CFA LUNG



c) MOUSE LUNG 3 WEEKS POST FITC

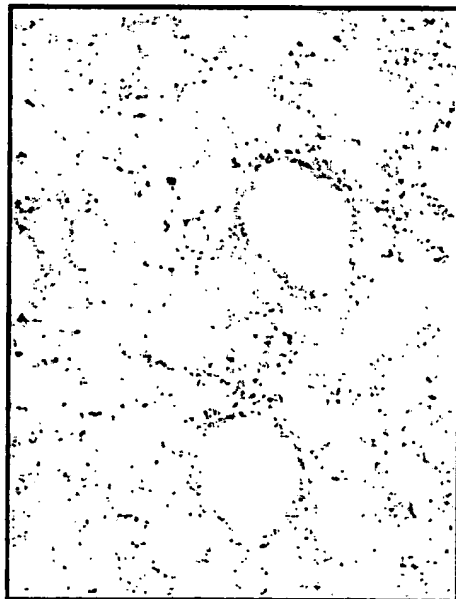
SUBSTITUTE SHEET (RULE 26)



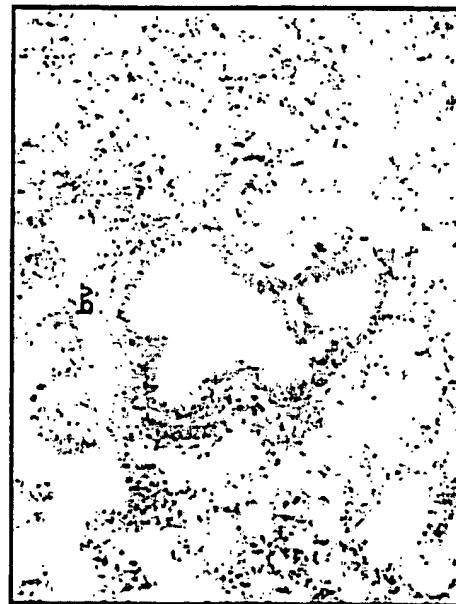
**FIG. 8**

Shh INDUCED EC HYPERPLASIA

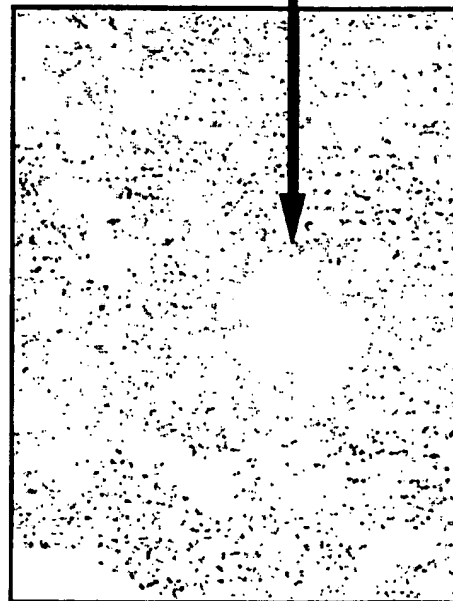
DAY 7 PBS



DAY 7 SPC



DAY 7 Shh



EC HYPERPLASIA



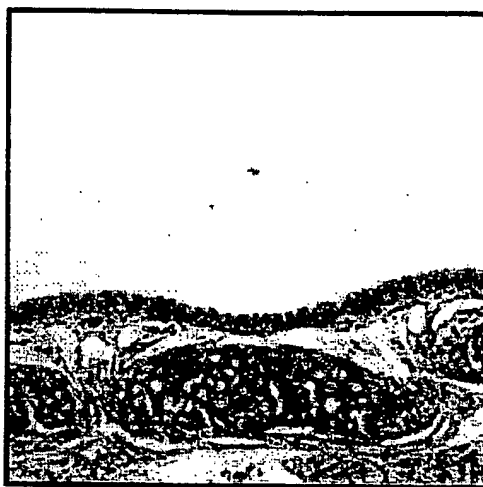
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FIG. 9  
TRACHEA

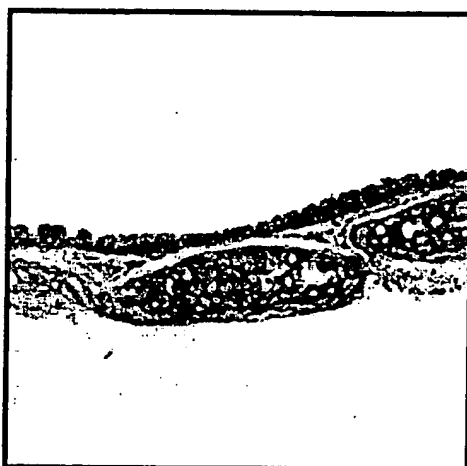
DAY 30 Shh



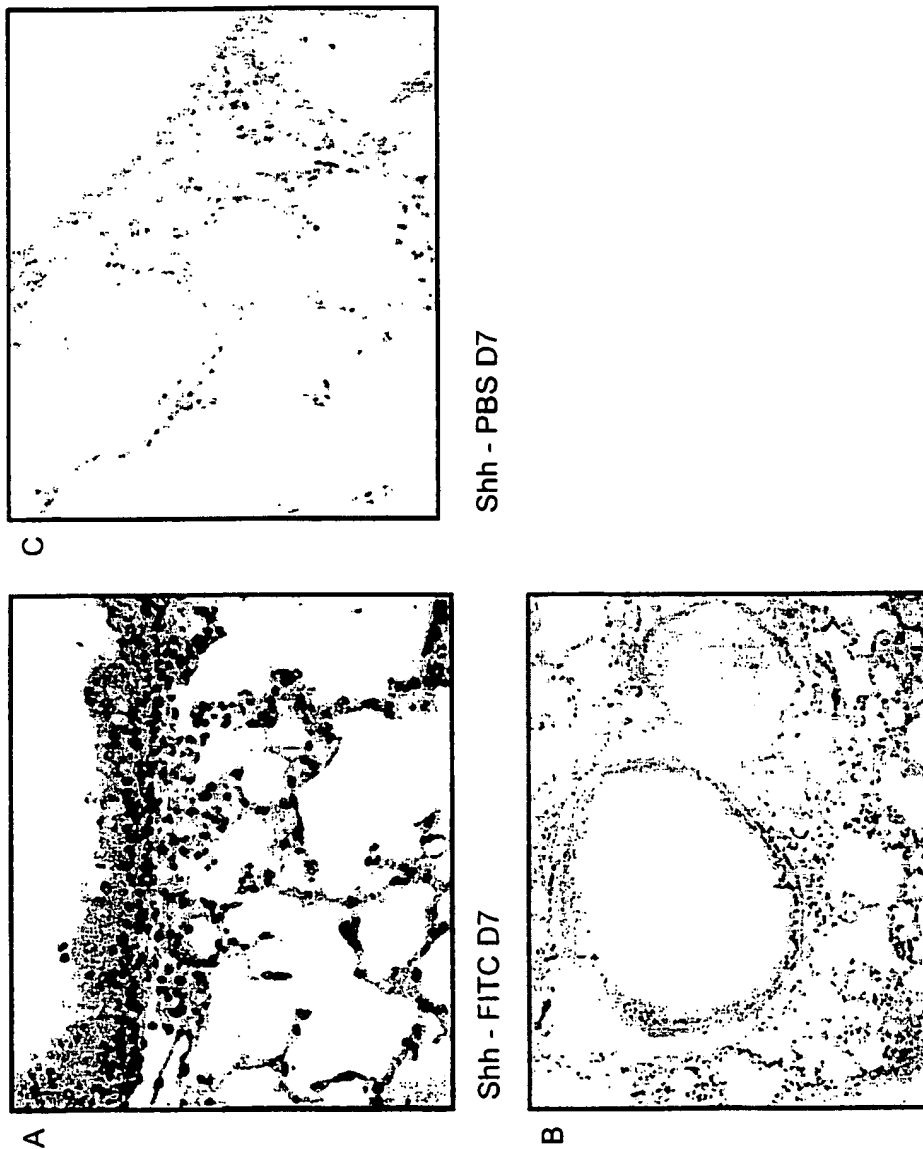
DAY 30 SPC

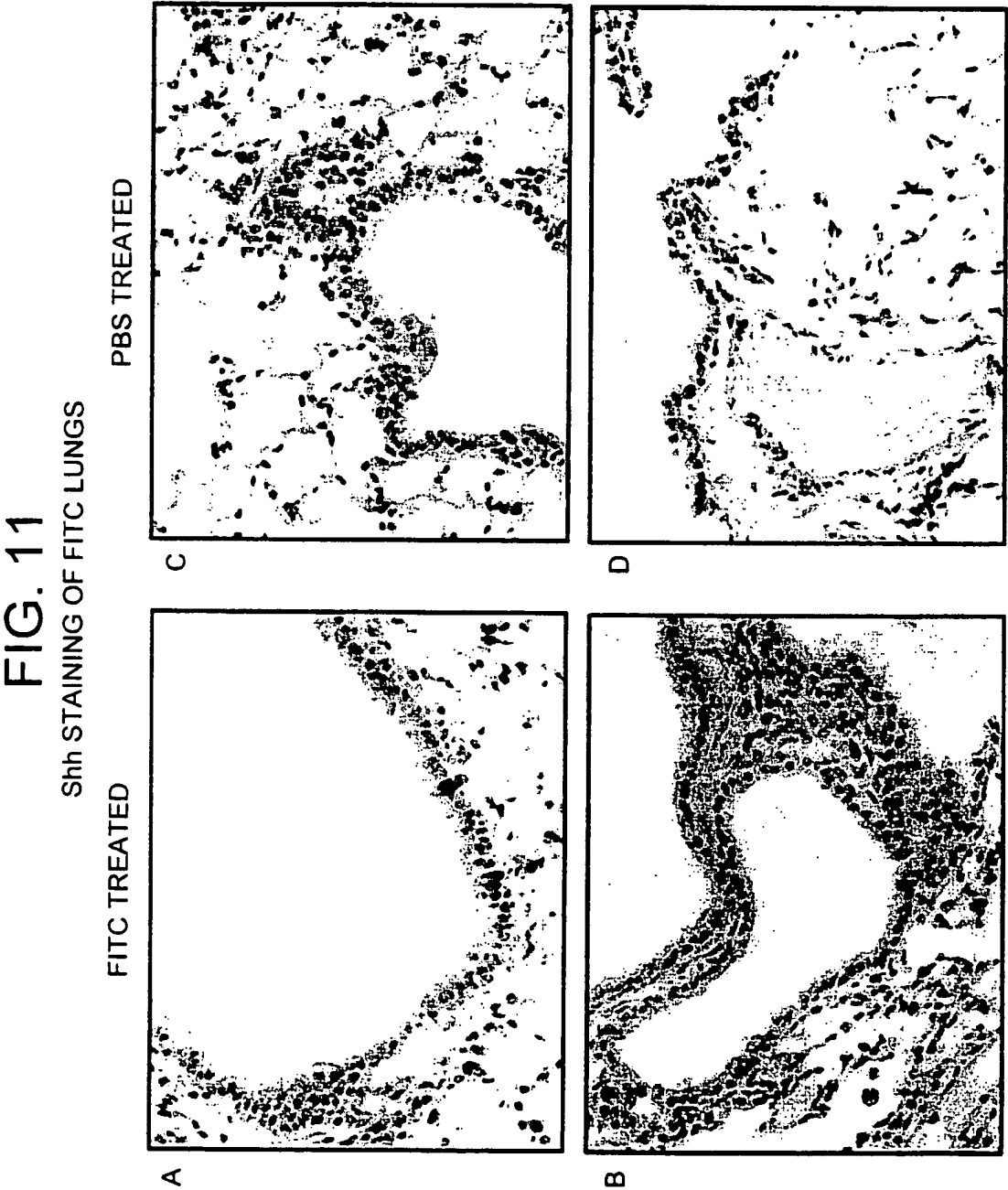


DAY 30 PBS



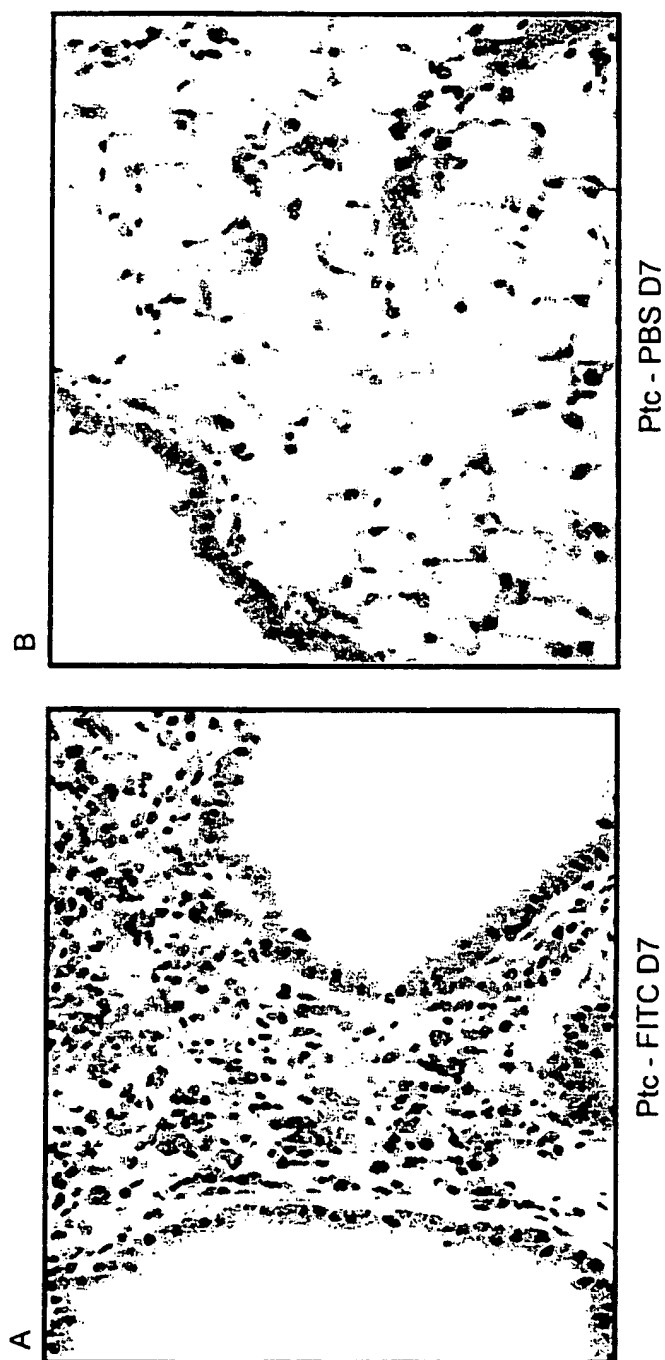
**FIG. 10**  
Shh STAINING OF FITC LUNGS





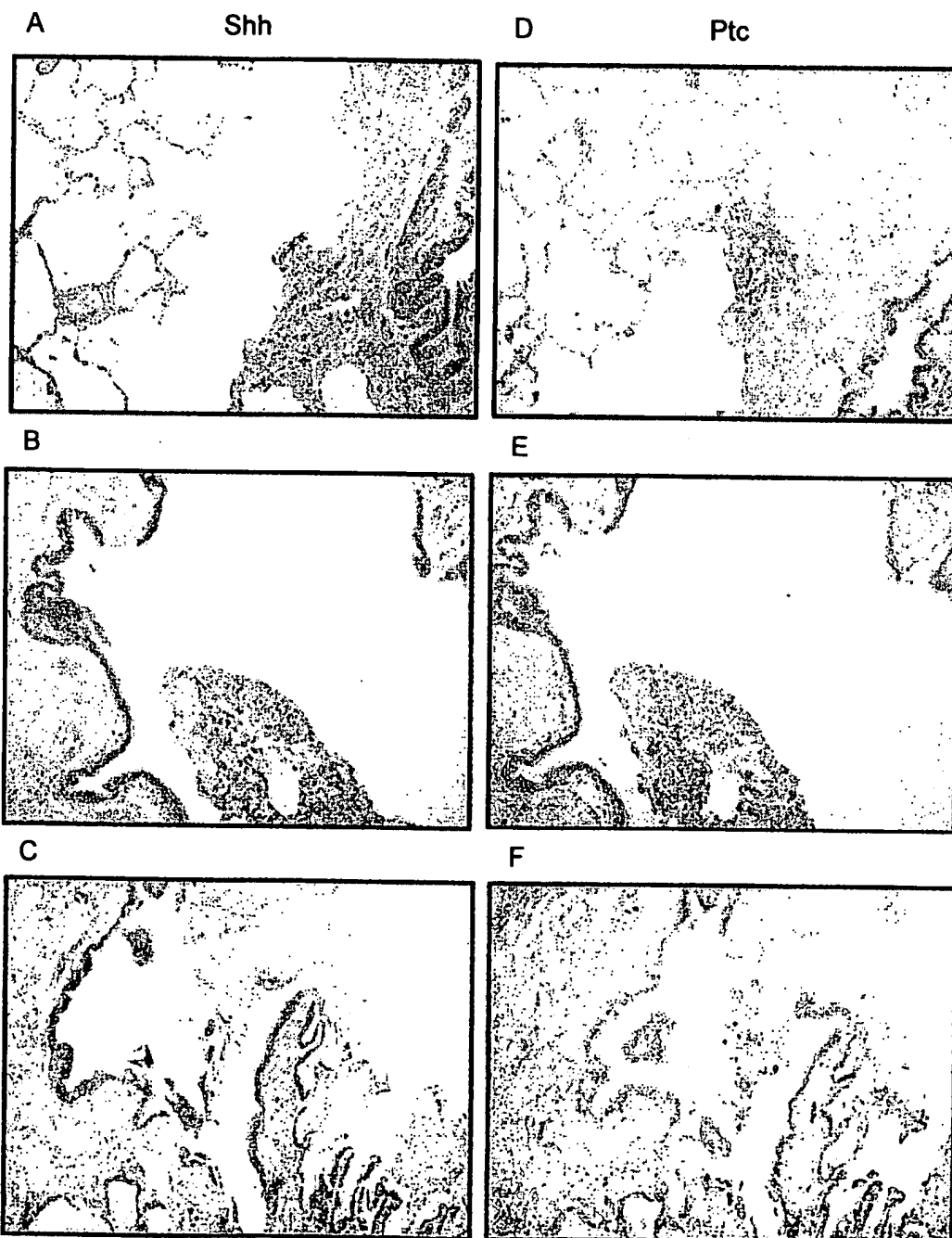


**FIG. 12**  
Ptc STAINING OF FITC LUNGS



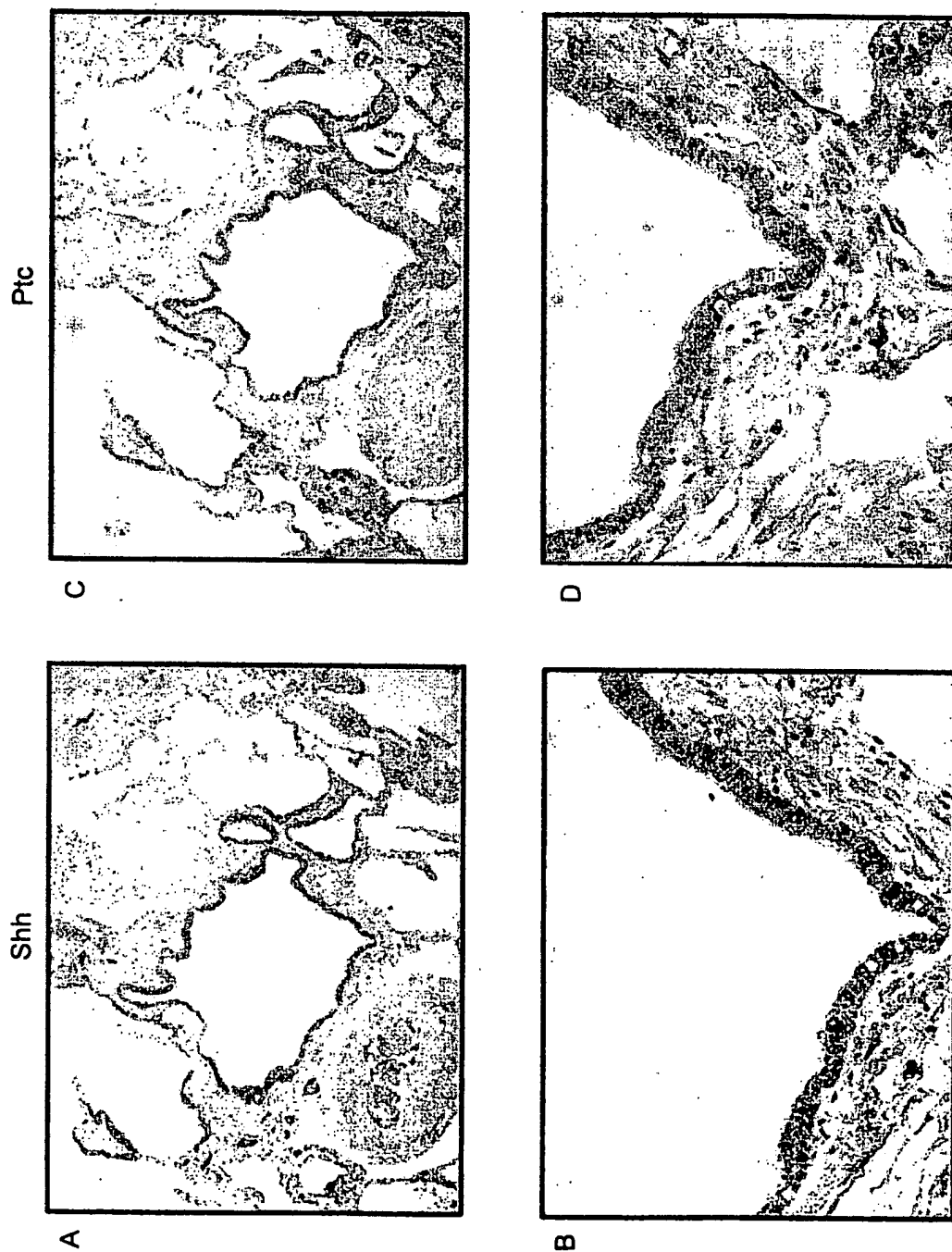
# FIG. 13

CFA PATIENT



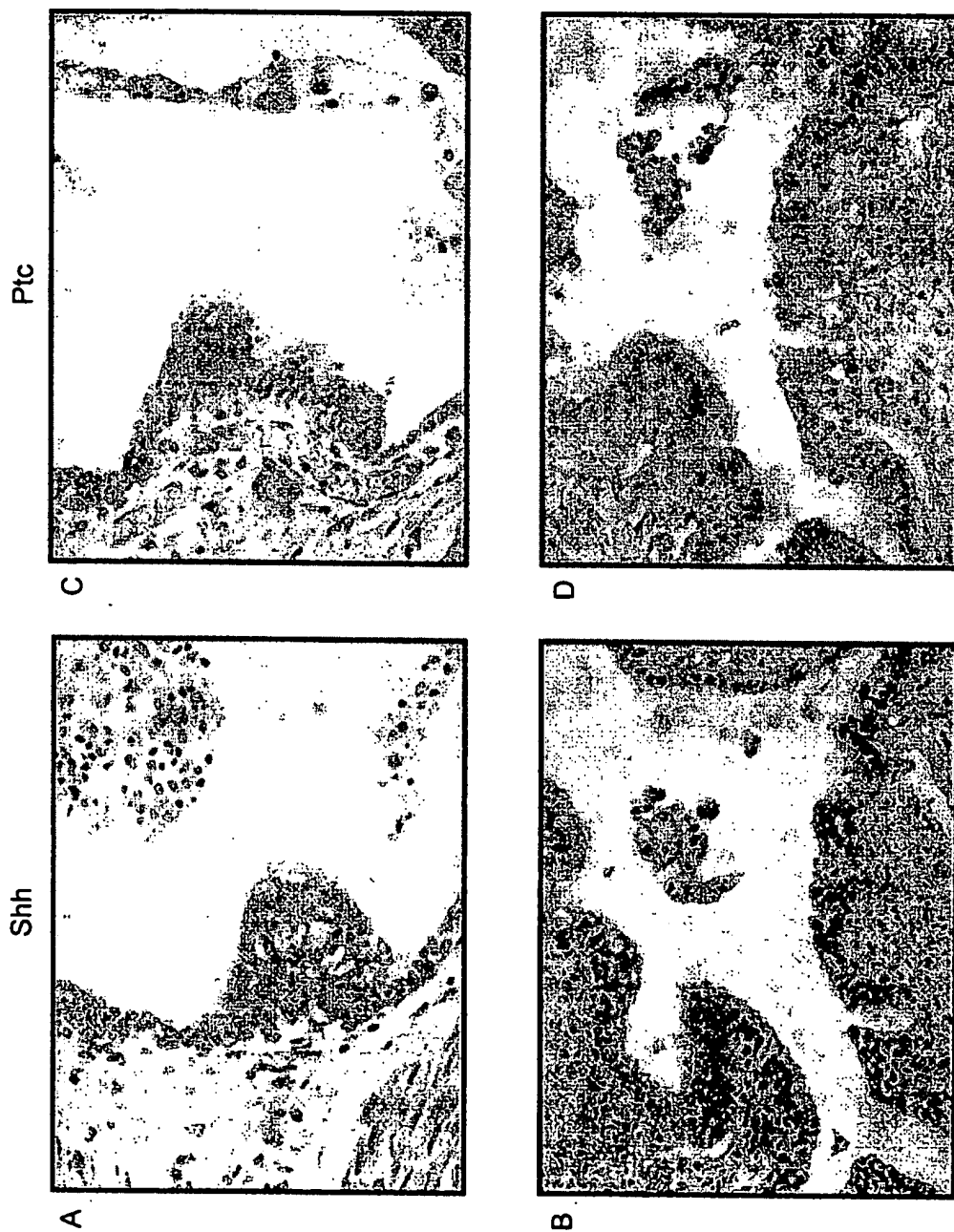
**FIG. 14**

CFA PATIENT

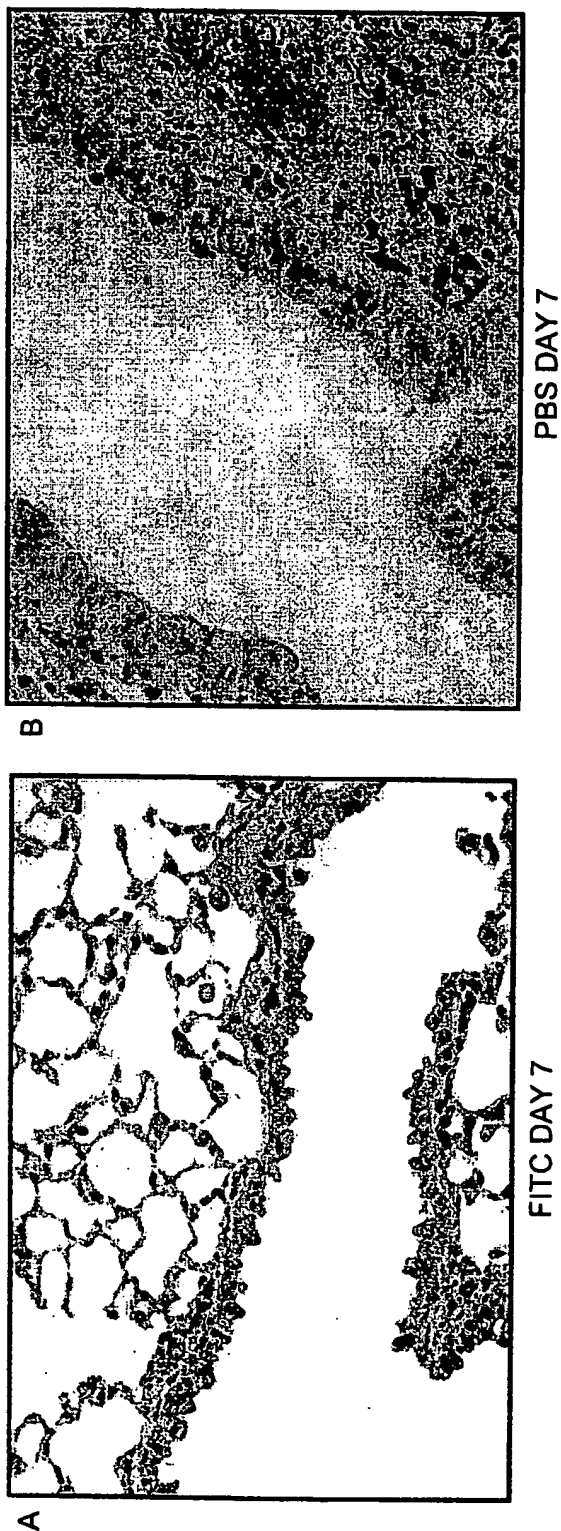


**FIG. 15**

CFA PATIENT

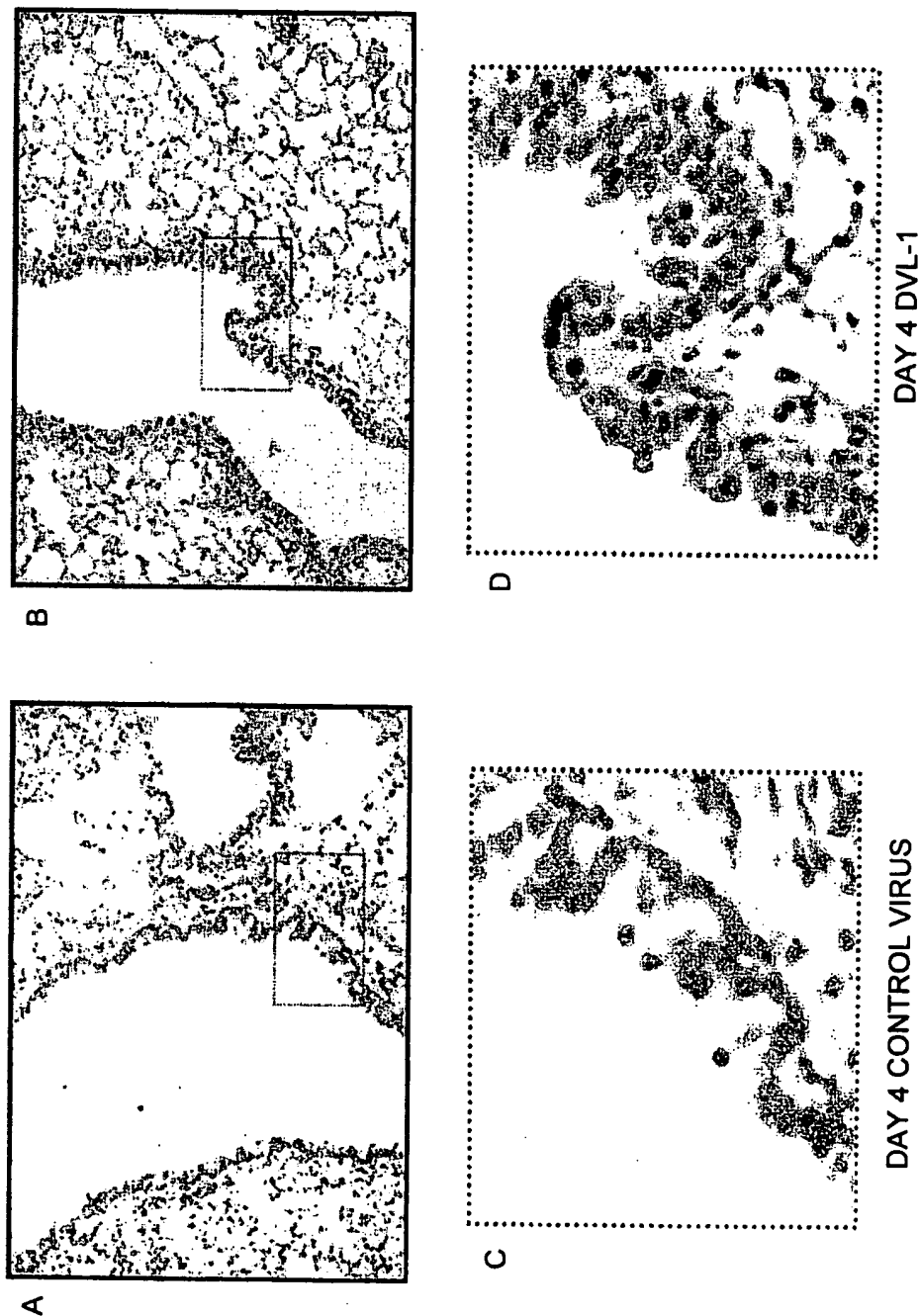


**FIG. 16**  
DVL-1 STAINING OF FITC LUNGS



**FIG. 17**

DVL 1 ADENOVIRUS *in vivo* (Ki67)



## SEQ ID NOS: 1 &amp; 2

DEFINITION M.musculus (C57BL/6J) Shh mRNA.  
 ACCESSION X76290  
 NID g2597987  
 KEYWORDS shh gene; sonic hedgehog protein.  
 SOURCE house mouse.  
 ORGANISM <a href=/htbin-post/Taxonomy/wgetorg?id=10090>Mus musculus</a>  
 Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;  
 Rodentia; Sciurognathi; Muridae; Murinae; Mus.  
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## SEQ ID NOS: 3 &amp; 4

DEFINITION Mus musculus C57 Black/6 dishevelled segment polarity protein homolog (Dvl-1) mRNA, complete cds.

ACCESSION U10115

NID g497689

KEYWORDS .

SOURCE mouse.

ORGANISM <a href="/htbin-post/Taxonomy/wgetorg?id=10090">Mus musculus</a>  
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Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

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BASE COUNT 679 a 969 c 1013 g 625 t

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## SEQ ID NOS: 5 &amp; 6

DEFINITION Mus musculus hedgehog-interacting protein (Hip) mRNA, complete cds.  
 ACCESSION AF116865  
 NID g4868121  
 KEYWORDS .  
 SOURCE house mouse.  
 ORGANISM <a href=/htbin-post/Taxonomy/wgetorg?id=10090>Mus musculus</a>  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;  
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BASE COUNT 660 a 722 c 672 g 615 t

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## SEQ ID NOS: 7 &amp; 8

DEFINITION Mus musculus Wnt inhibitory factor-1 mRNA, complete cds.  
 ACCESSION AF122923  
 NID g4585371  
 KEYWORDS .  
 SOURCE house mouse.  
 ORGANISM [Mus musculus](/htbin-post/Taxonomy/wgetorg?id=10090)  
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# INTERNATIONAL SEARCH REPORT

1. national Application No

PCT/GB 00/02191

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61K39/395 A61K48/00 G01N33/53 C12N15/63  
A01K67/027 A61P11/00 A61P13/12 A61P35/00 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. DUMONTELLE ET AL.: "Inhibition of expression of the endogenous mammary oncogene Wnt-1 by antisense oligodeoxynucleotides." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 36, March 1995 (1995-03), page 515 XP002147584 USA abstract 3065 --- -/--	1,3, 12-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

15 September 2000

Date of mailing of the international search report

04/10/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3018

Authorized officer

Nooij, F

# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/GB 00/02191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E. FUJITA ET AL.: "Involvement of sonic hedgehog in the cell growth of LK-2 cells, human lung squamous carcinoma cells." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 238, no. 2, 18 September 1997 (1997-09-18), pages 658-664, XP002147585 Duluth, MN, USA abstract figure 5	1,2,6,7, 12-14
X	WO 99 22000 A (DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 6 May 1999 (1999-05-06) the whole document	1,3,6,9, 12-15
X	M. MURONE ET AL.: "Sonic hedgehog signaling by the patched-smoothened receptor complex." CURRENT BIOLOGY, vol. 9, no. 2, 28 January 1999 (1999-01-28), pages 76-84, XP000929784 the whole document	1-3,5, 13-15
X	J. POLANEC ET AL.: "Effect of Wnt-1 antisense RNA on the outgrowth of a mammary adenocarcinoma cell line expressing that oncogene." CLINICAL MOLECULAR PATHOLOGY, vol. 49, no. 3, 1996, pages M166-M169, XP000929749 abstract	1,3, 12-16
A	EP 0 874 048 A (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) 28 October 1998 (1998-10-28) examples 4,5 claims	1-20
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National Application No

PCT/GB 00/02191

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US 20040131585A1

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(54) **IDENTIFICATION AND USE OF HUMAN  
BONE MARROW-DERIVED ENDOTHELIAL  
PROGENITOR CELLS TO IMPROVE  
MYOCARDIAL FUNCTION AFTER  
ISCHEMIC INJURY**

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(21) **Appl. No.: 10/220,554**

(22) **PCT Filed: Jun. 5, 2001**

(86) **PCT No.: PCT/US01/18399**

**Related U.S. Application Data**

(63) **Continuation-in-part of application No. 09/587,441,  
filed on Jun. 5, 2000, now abandoned.**

**Publication Classification**

(51) **Int. Cl.<sup>7</sup> ..... A61K 45/00; A61K 38/19;**

**A61K 38/20**

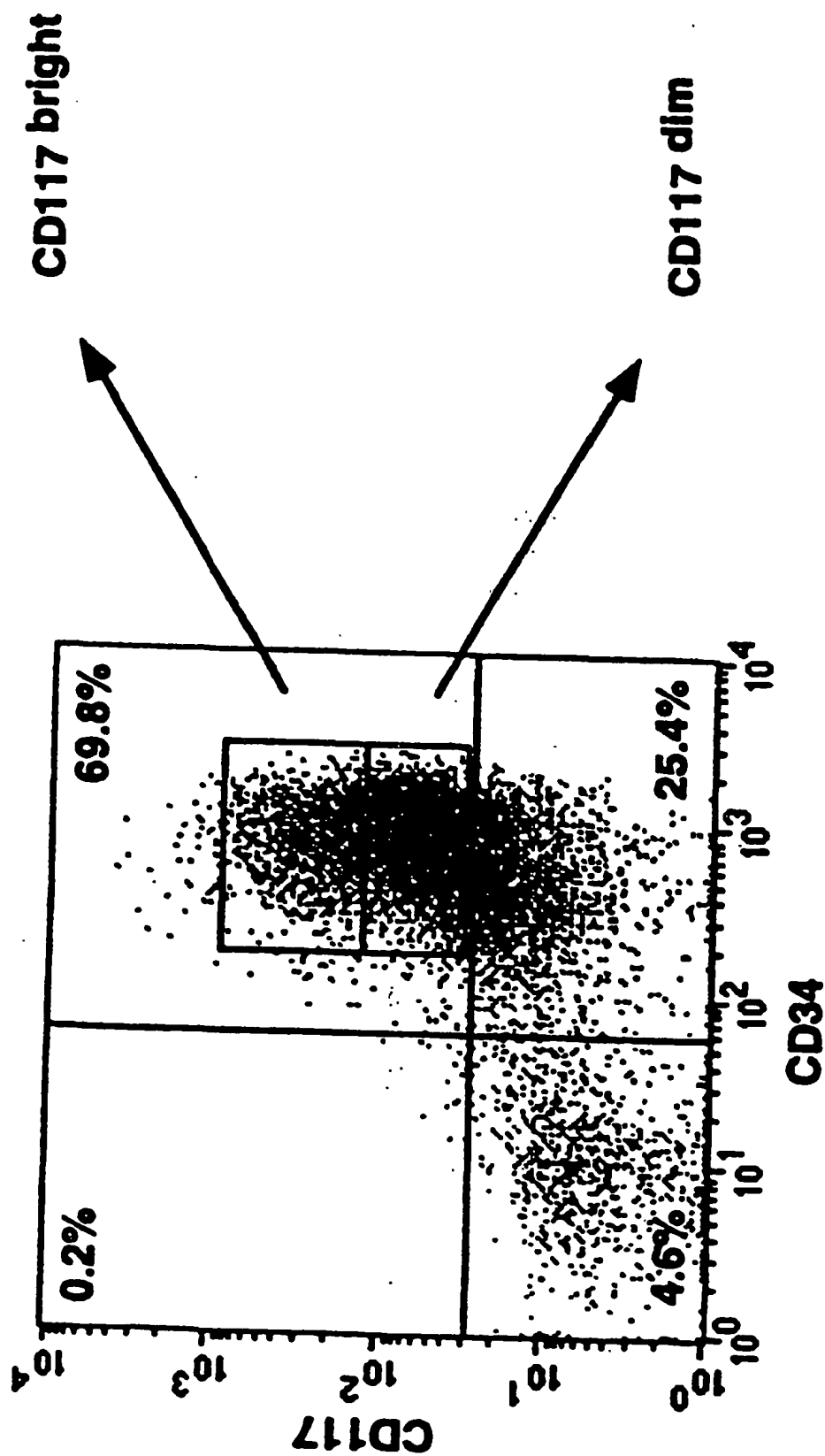
(52) **U.S. Cl. .... 424/85.1; 424/93.7**

(57) **ABSTRACT**

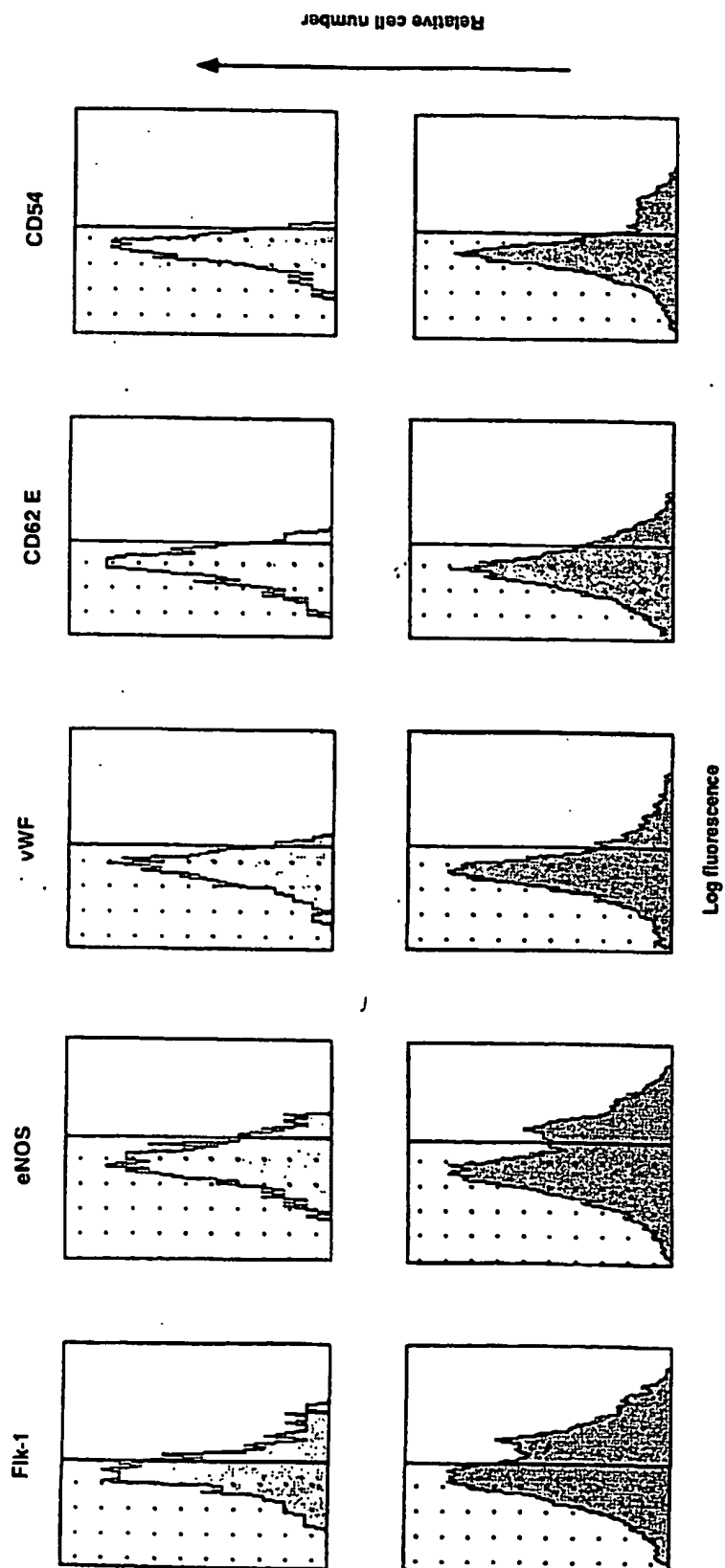
The present invention provides a method of stimulating vasculogenesis of myocardial infarct damaged tissue in a subject comprising: (a) removing stem cells from a location in the subject; (b) recovering endothelial progenitor cells in the stem cells; (c) introducing the endothelial progenitor cells from step (b) into a different location in the subject such that the precursors migrate to and stimulate revascularization of the tissue. The stem cells may be removed directly or by mobilization. The endothelial progenitor cells may be expanded before introduction into the subject. The present invention further provides a method of inducing angiogenesis in peri-infarct tissue. The present invention further provides a method of selectively increasing the trafficking of human bone marrow-derived endothelial cell precursors to the site of tissue damaged by ischemic injury which comprises: (a) administering endothelial progenitor cells to a subject; (b) administering chemokines to the subject so as to thereby attract endothelial cell precursors to the ischemic tissue. The present invention provides a method of stimulating vasculogenesis or angiogenesis of myocardial infarct damaged tissue in a subject comprising injecting allogeneic stem cells into a subject. The present invention further provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising any of the instant methods. The present invention further provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting G-CSF or anti-CXCR4 antibody into the subject in order to mobilize endothelial progenitor cells.



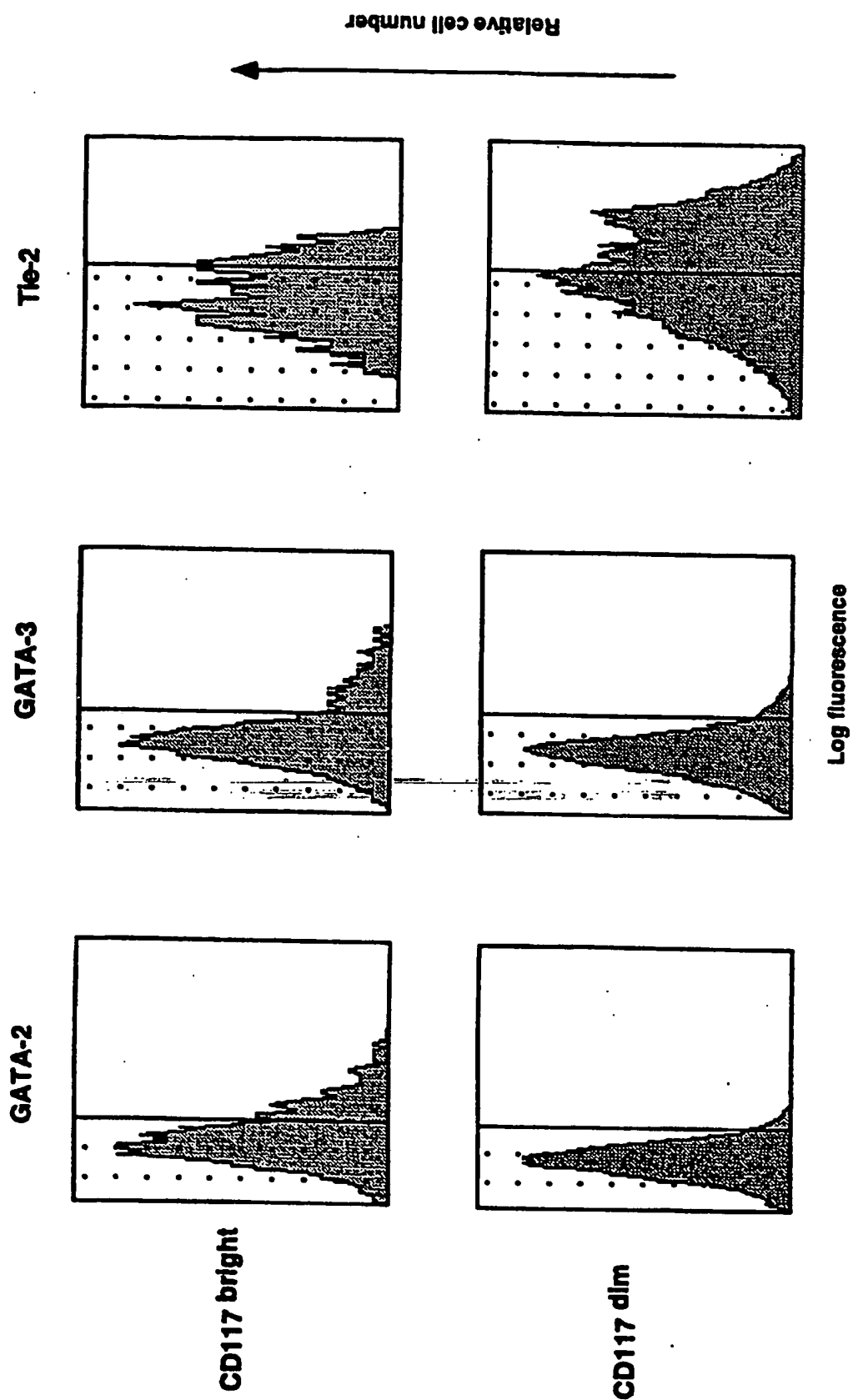
**FIGURE 1A**



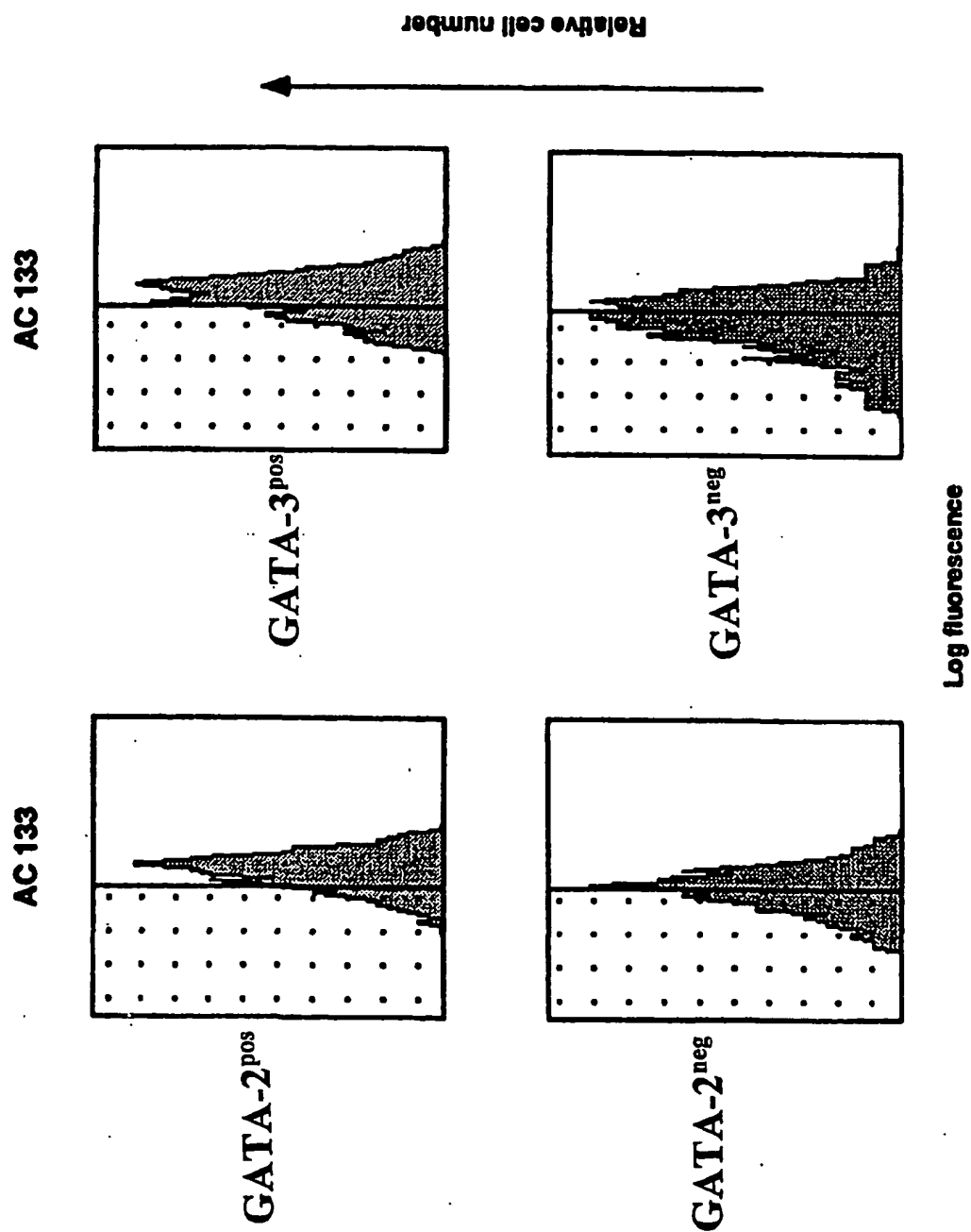
**FIGURE 1B**



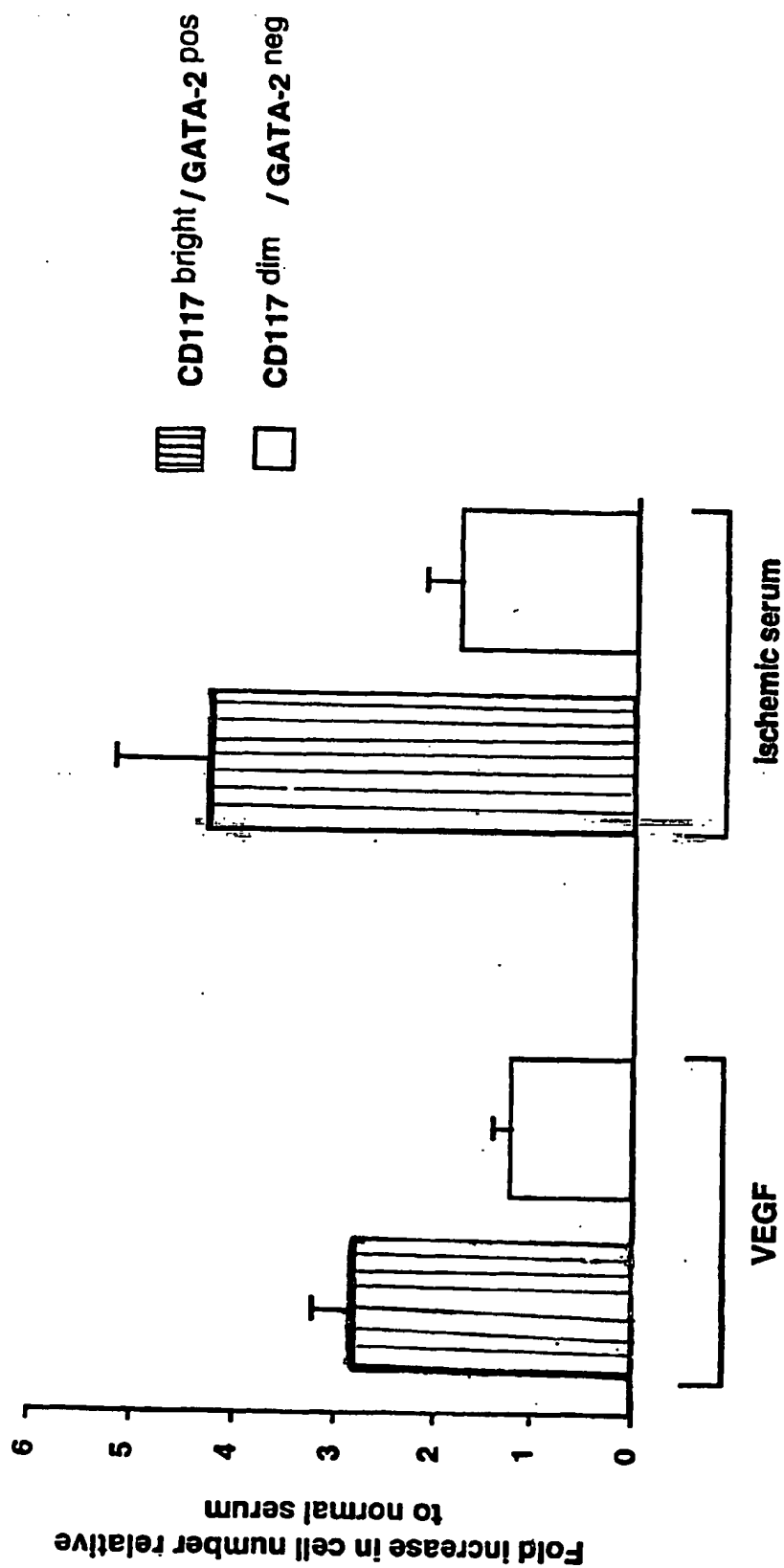
**FIGURE 1C**



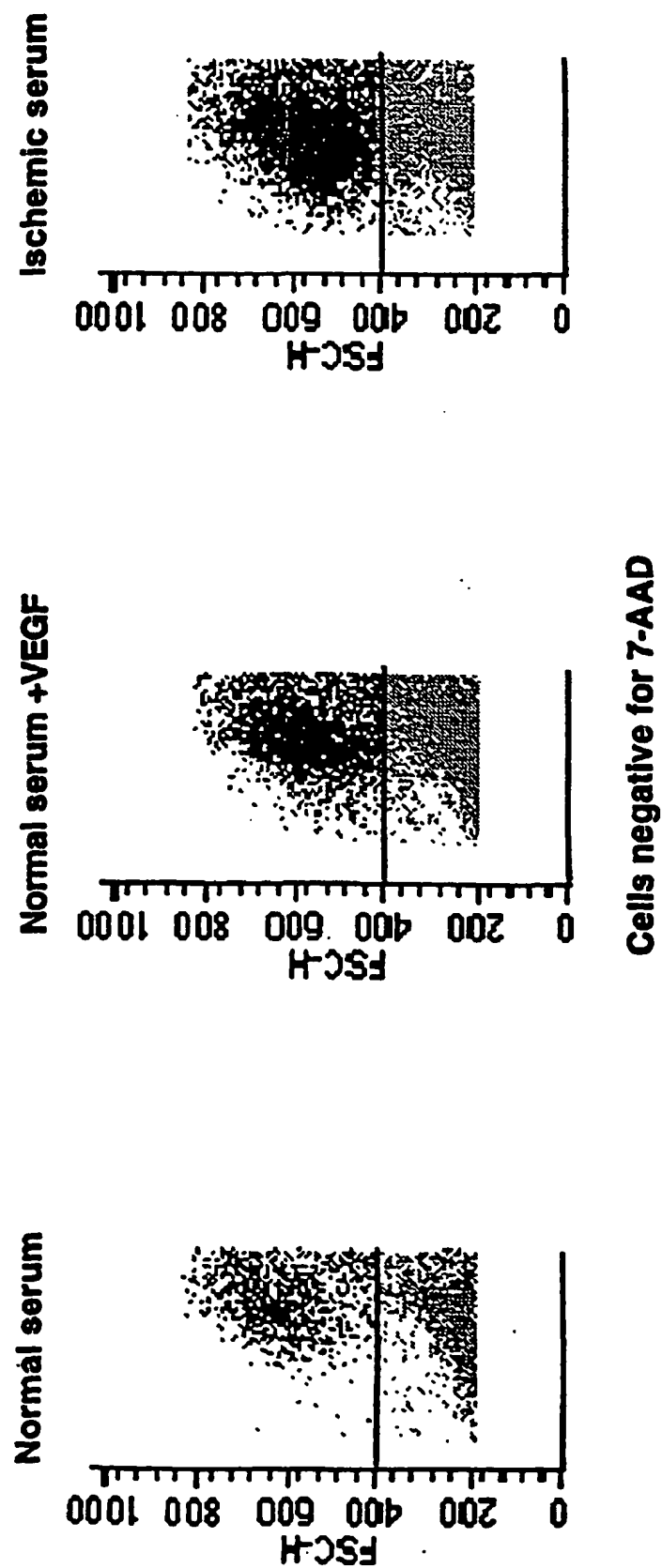
**FIGURE 1D**



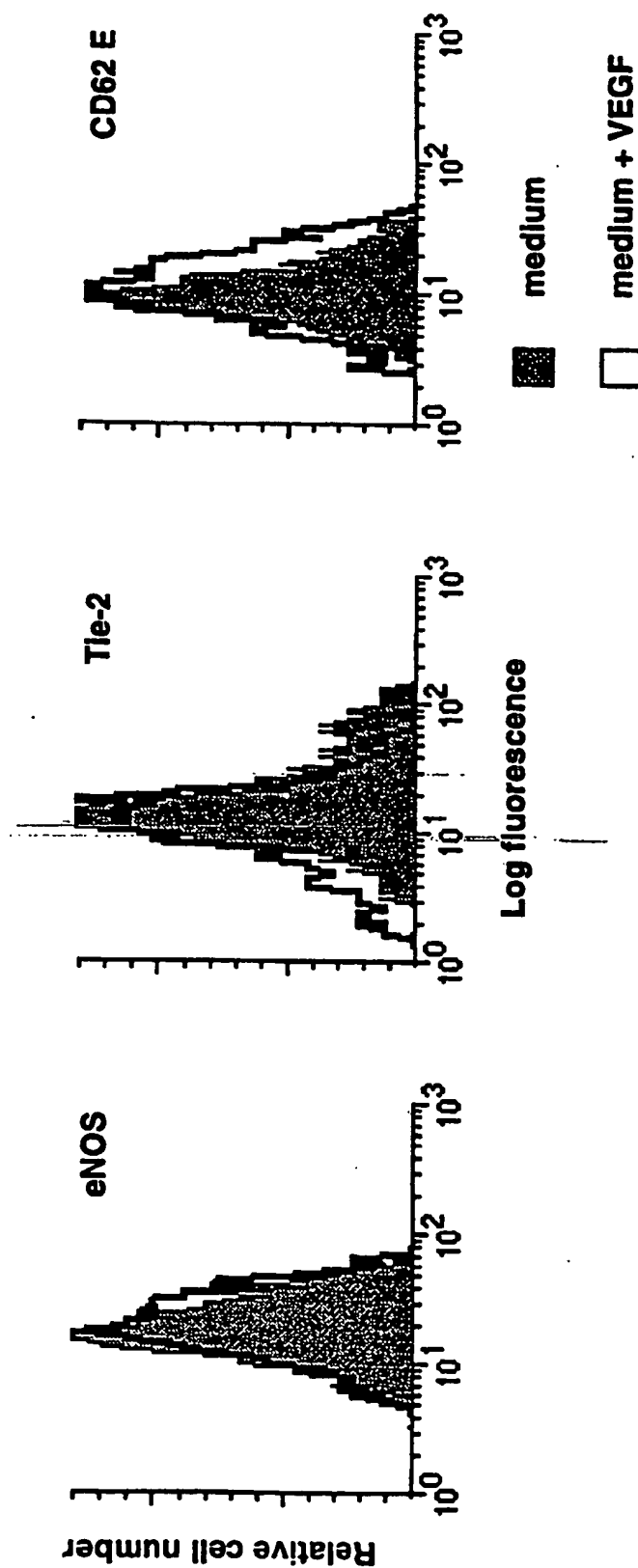
**FIGURE 2A**



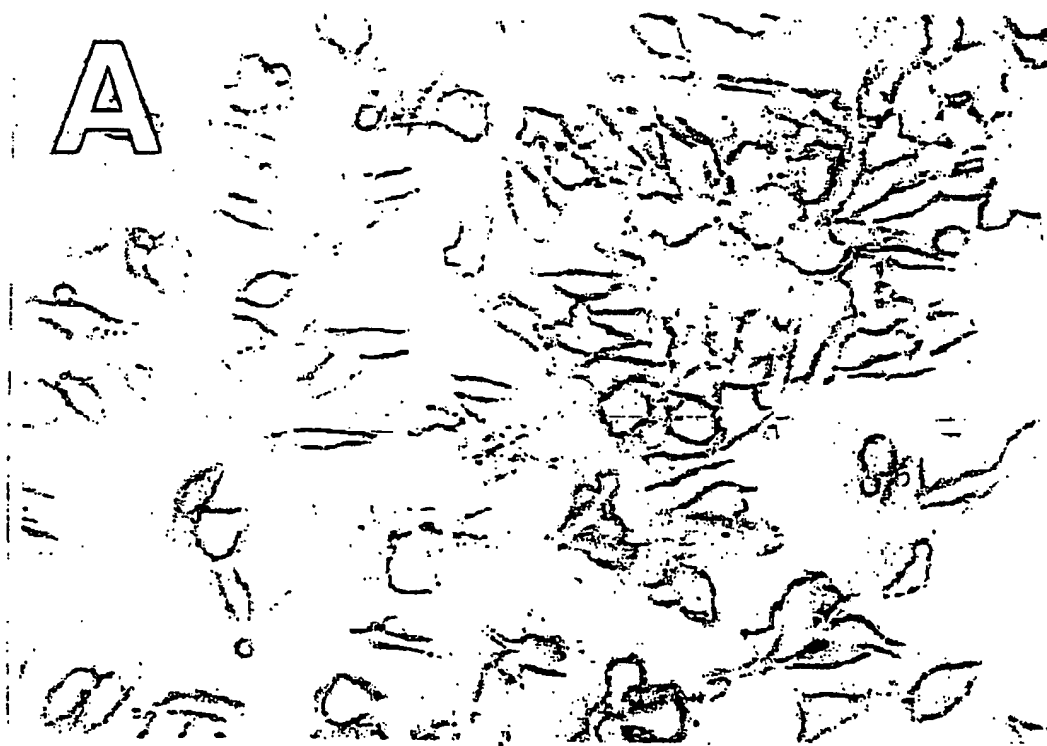
**FIGURE 2B**



**FIGURE 2C**

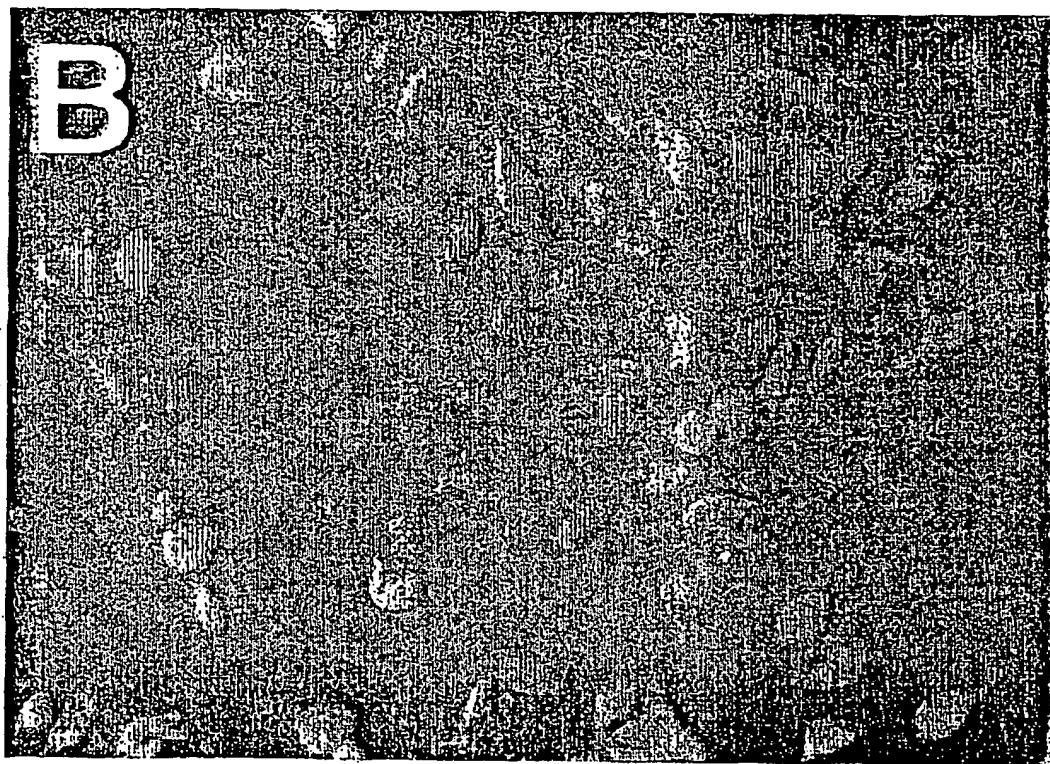


## FIGURE 3A

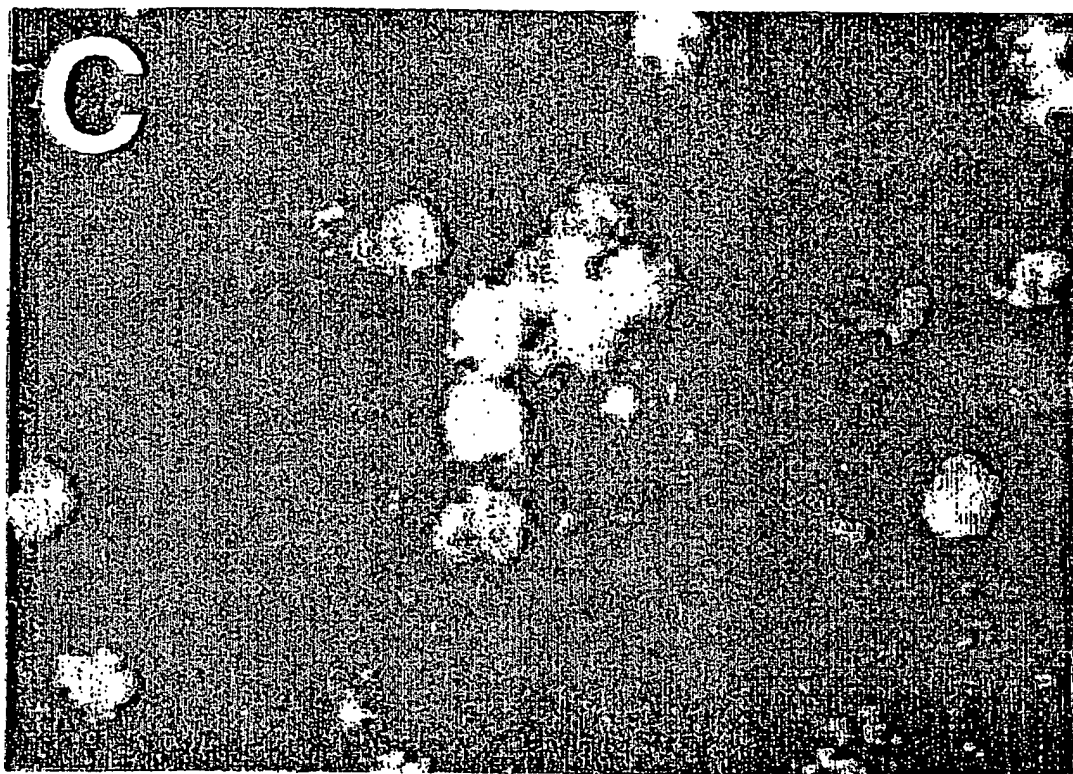




## FIGURE 3B



## FIGURE 3C



## FIGURE 3D

D

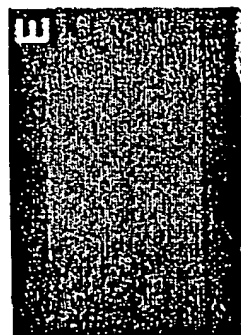
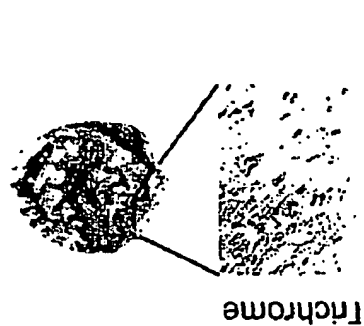


# FIGURE 3E

E



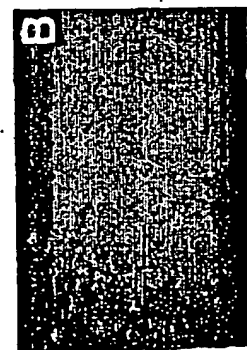
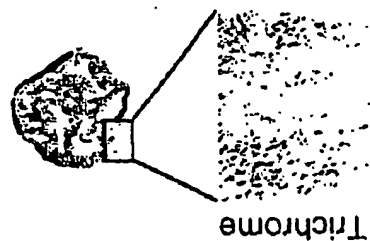
Sham procedure



CD34 +

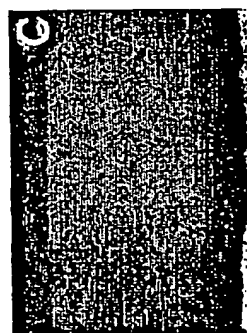
FIGURE 4D

Myocardial infarction



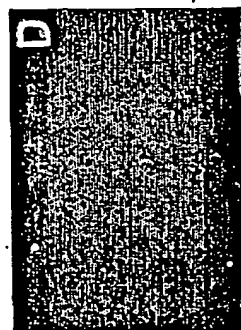
CD34 +

FIGURE 4A



CD34 -

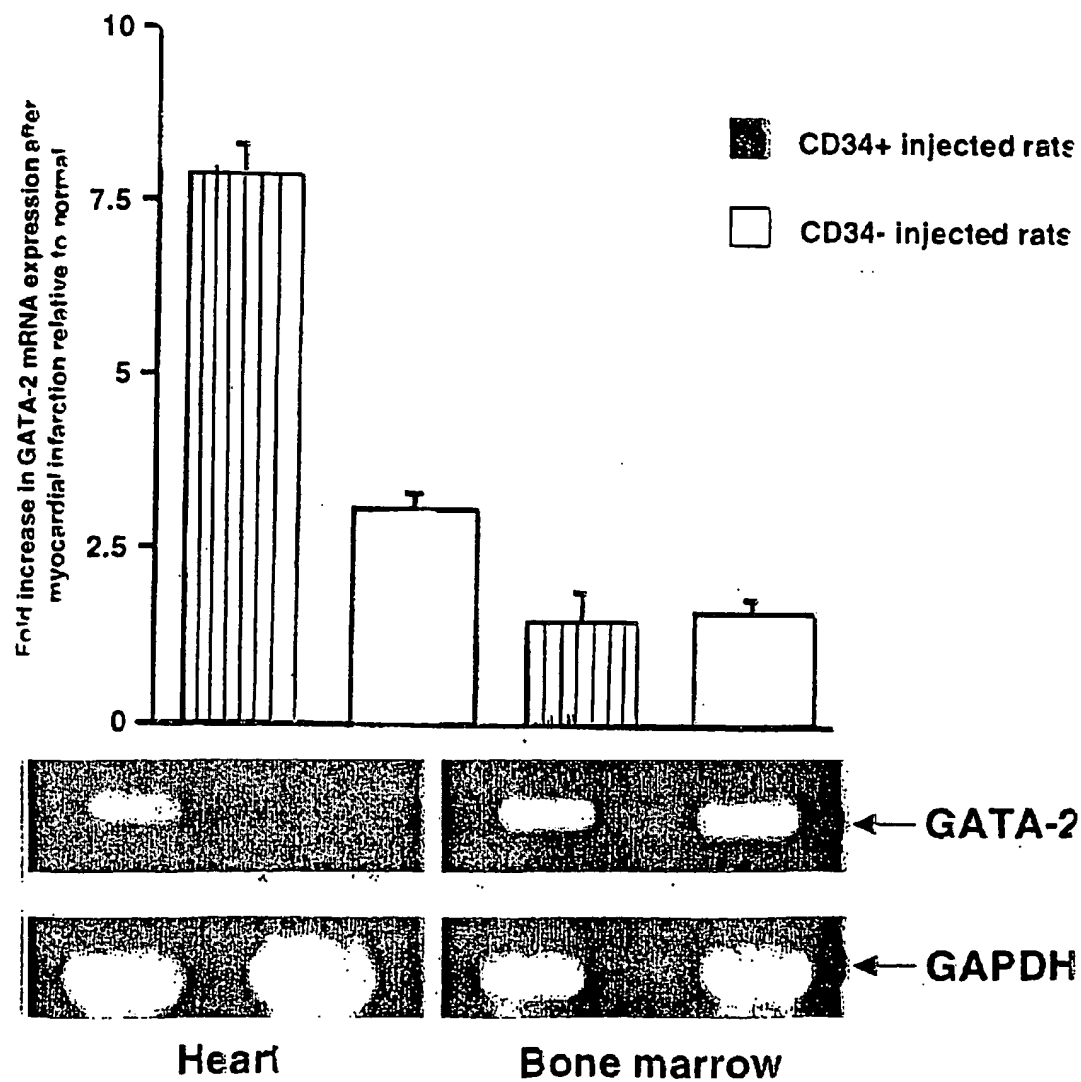
FIGURE 4B



SVEC

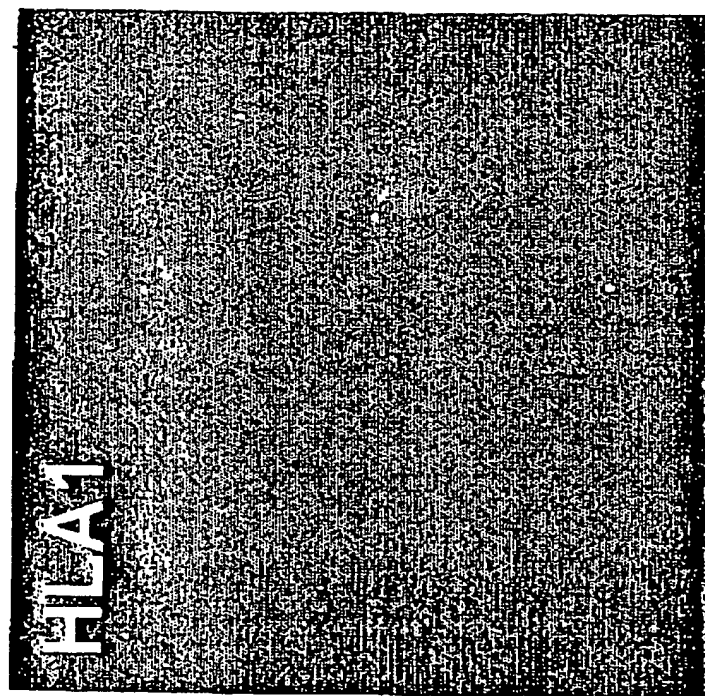
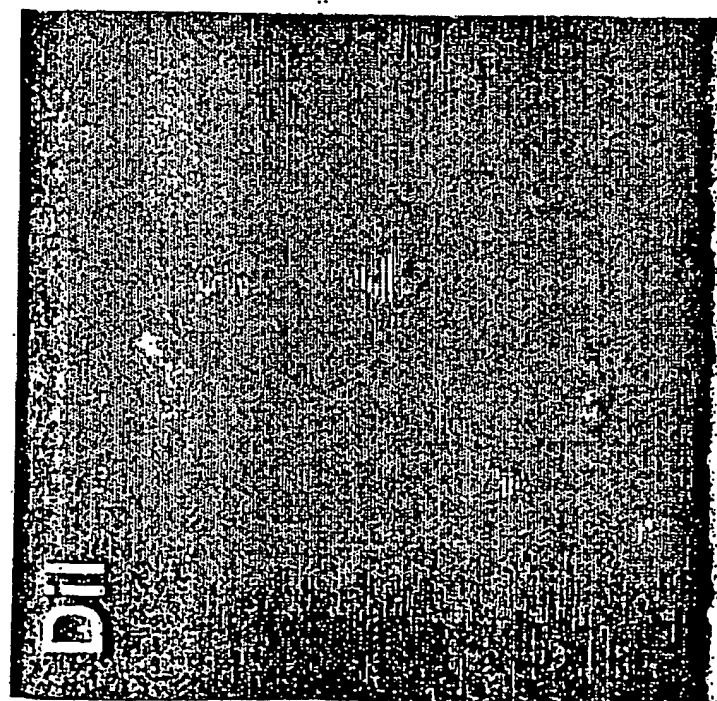
FIGURE 4C

# FIGURE 4E



# FIGURE 4F

F



FVIII

FIGURE 5A

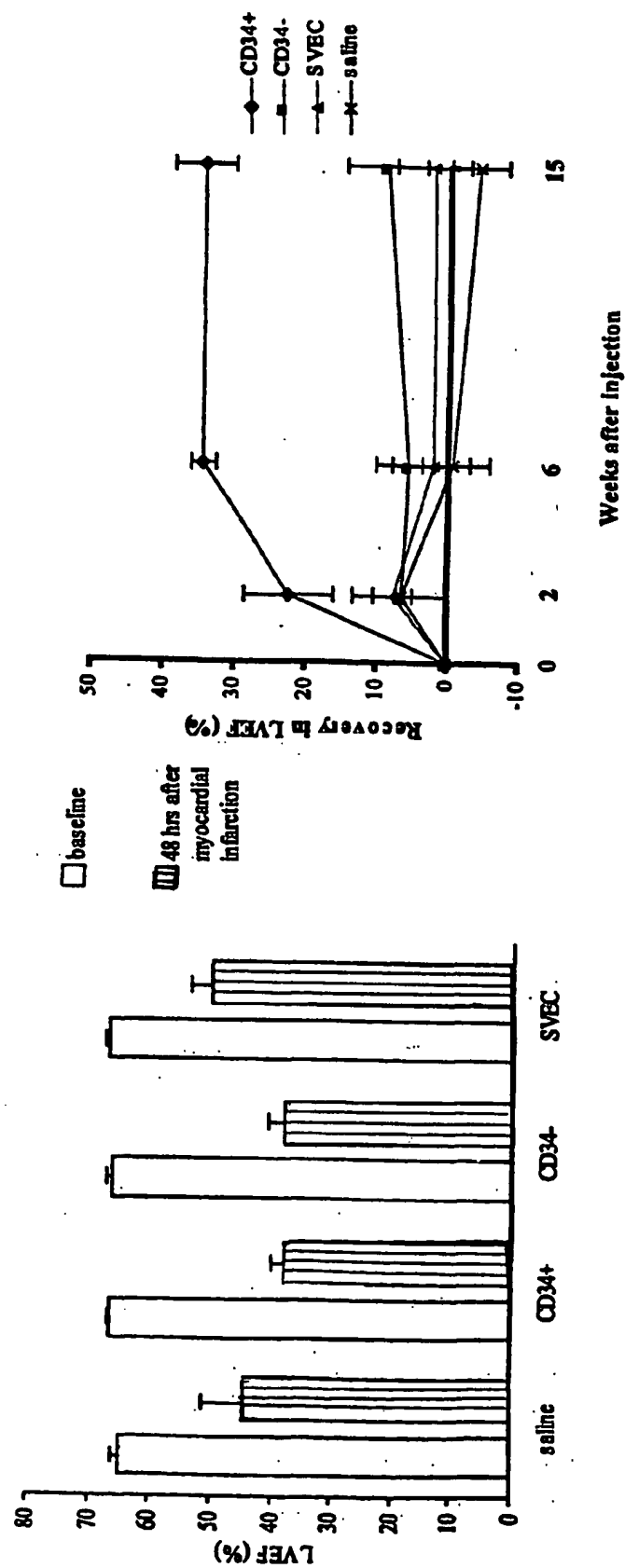




FIGURE 5B

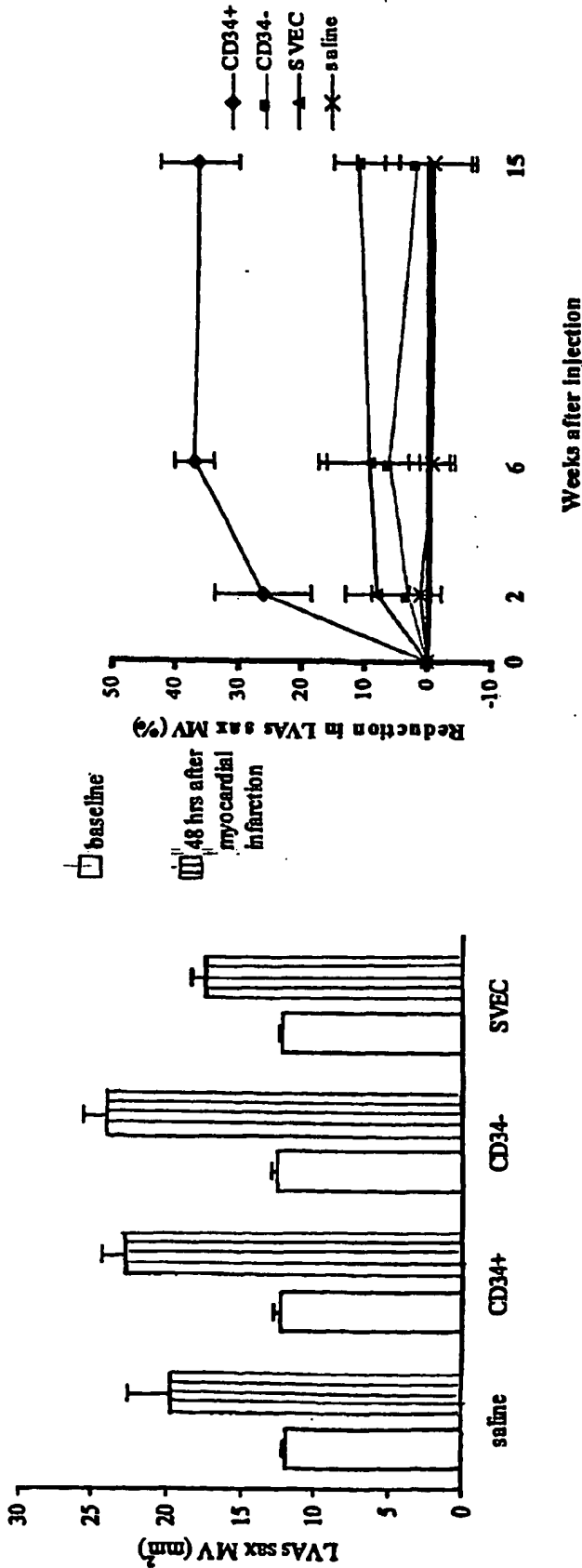
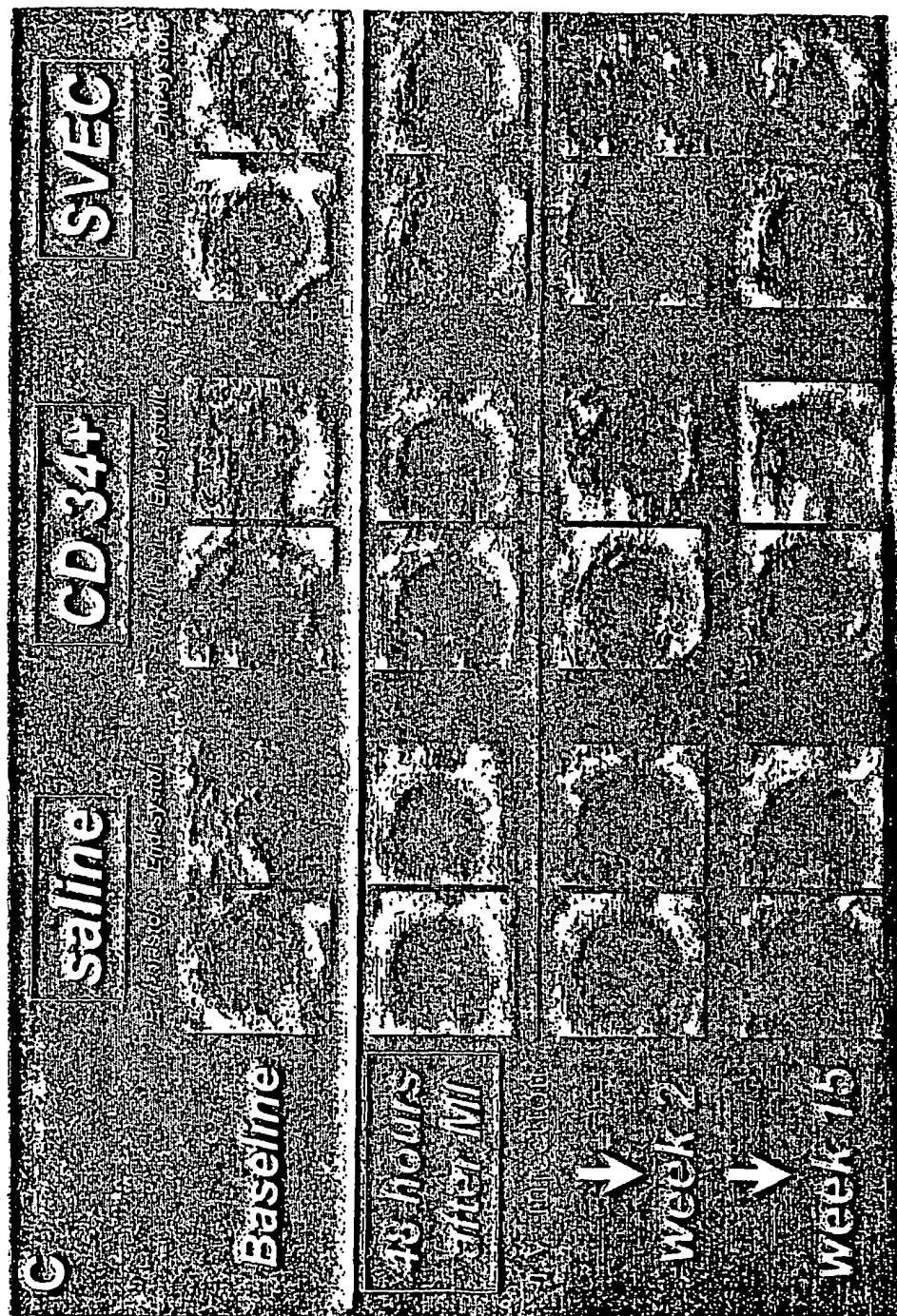
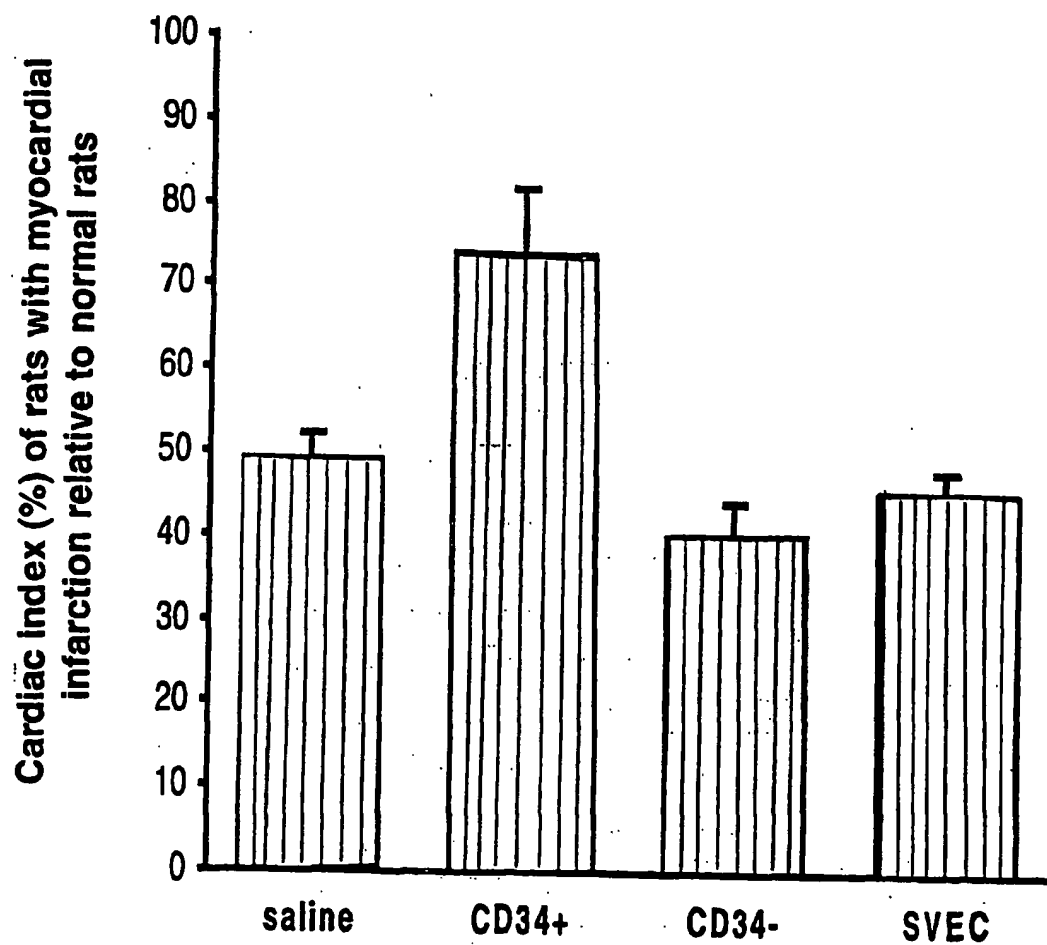


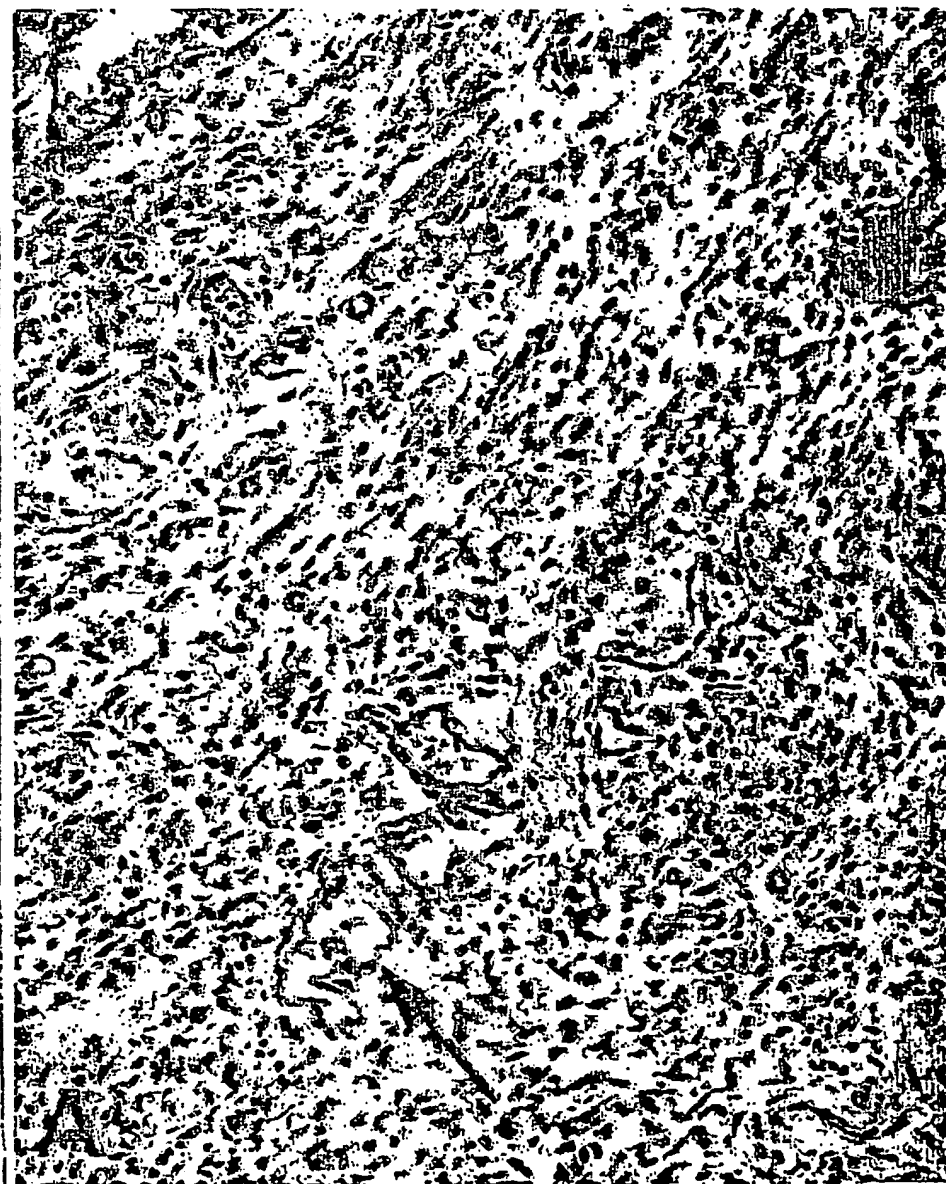
FIGURE 5C



## FIGURE 5D



# FIGURE 6A



# FIGURE 6B



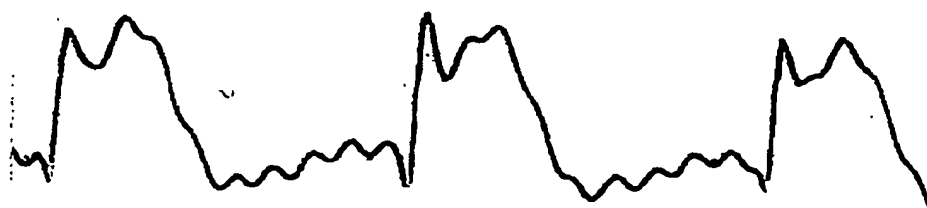
# FIGURE 6C



# FIGURE 6D



# FIGURE 6E

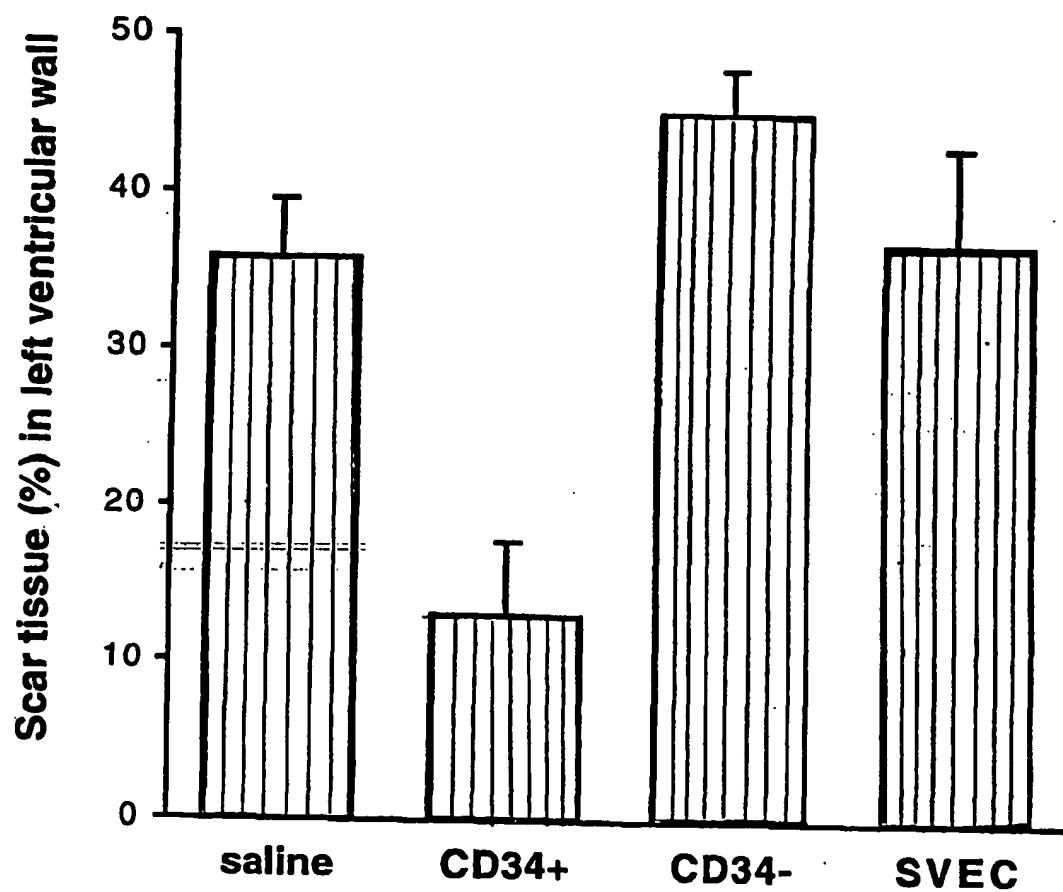


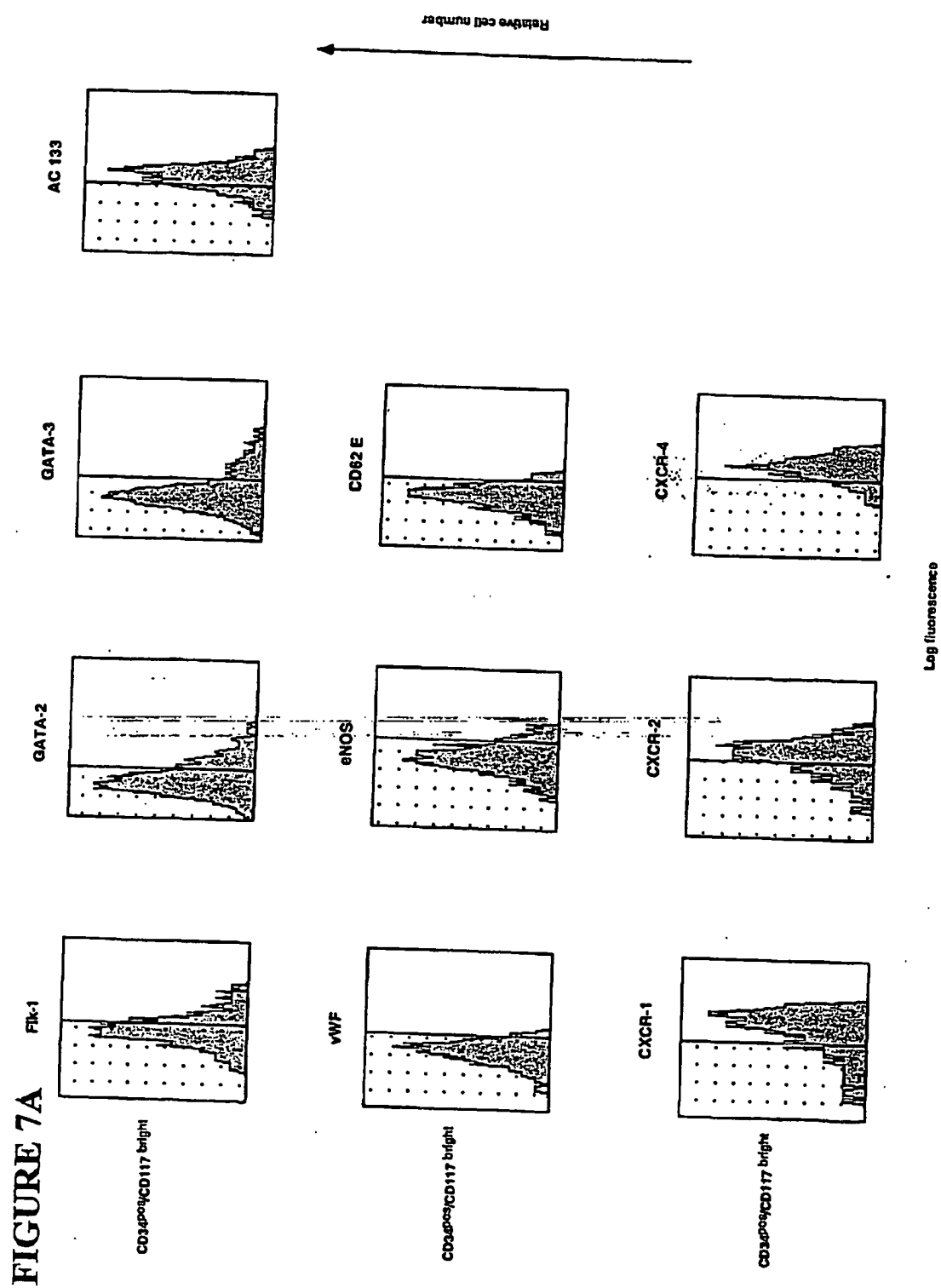


# FIGURE 6F

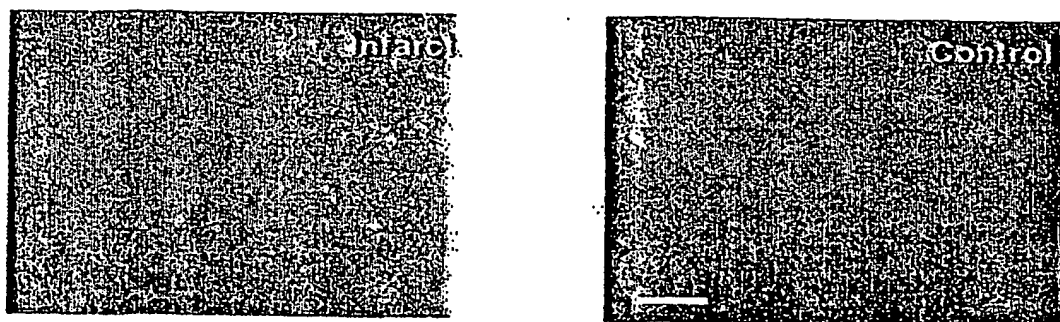


## FIGURE 6G

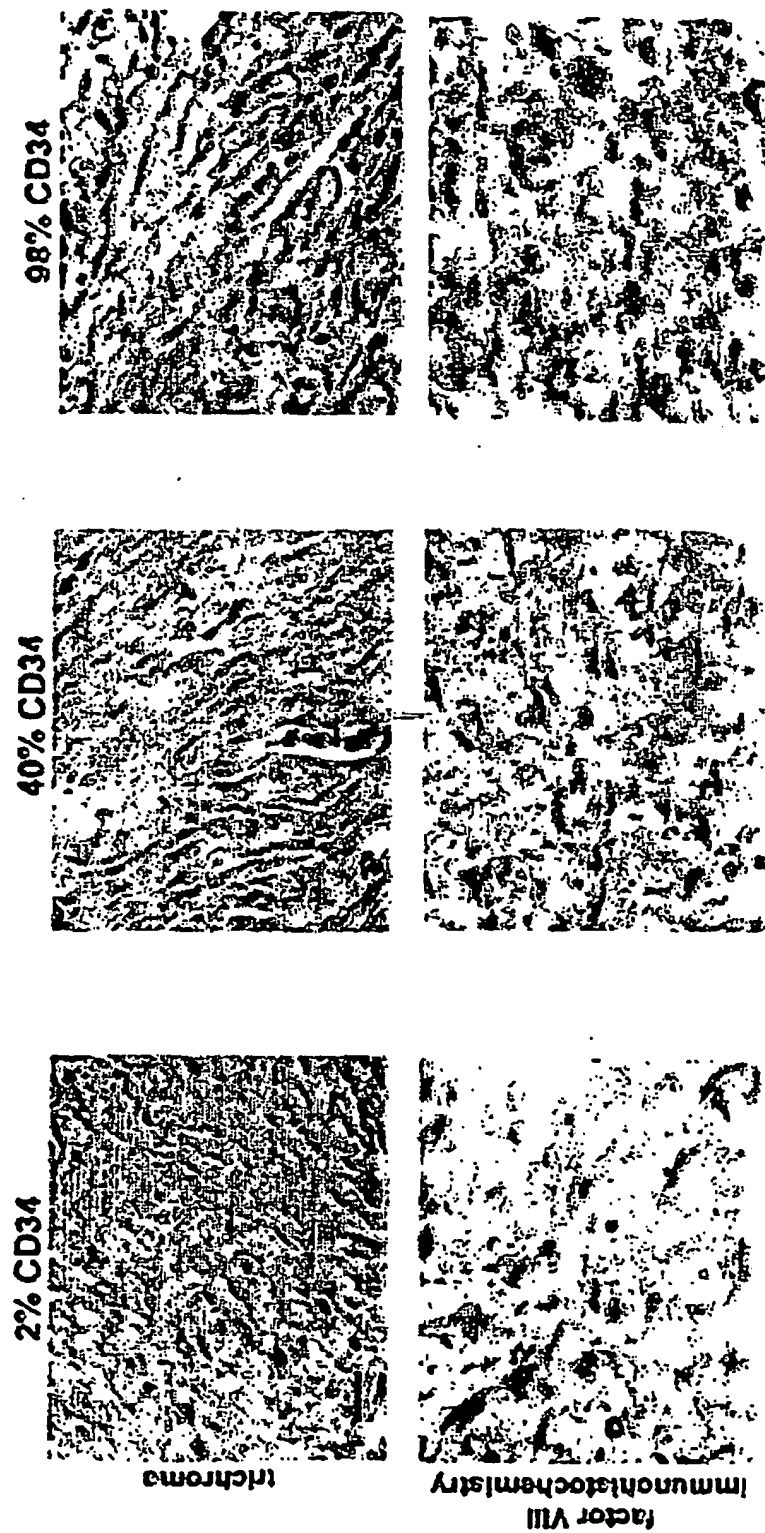




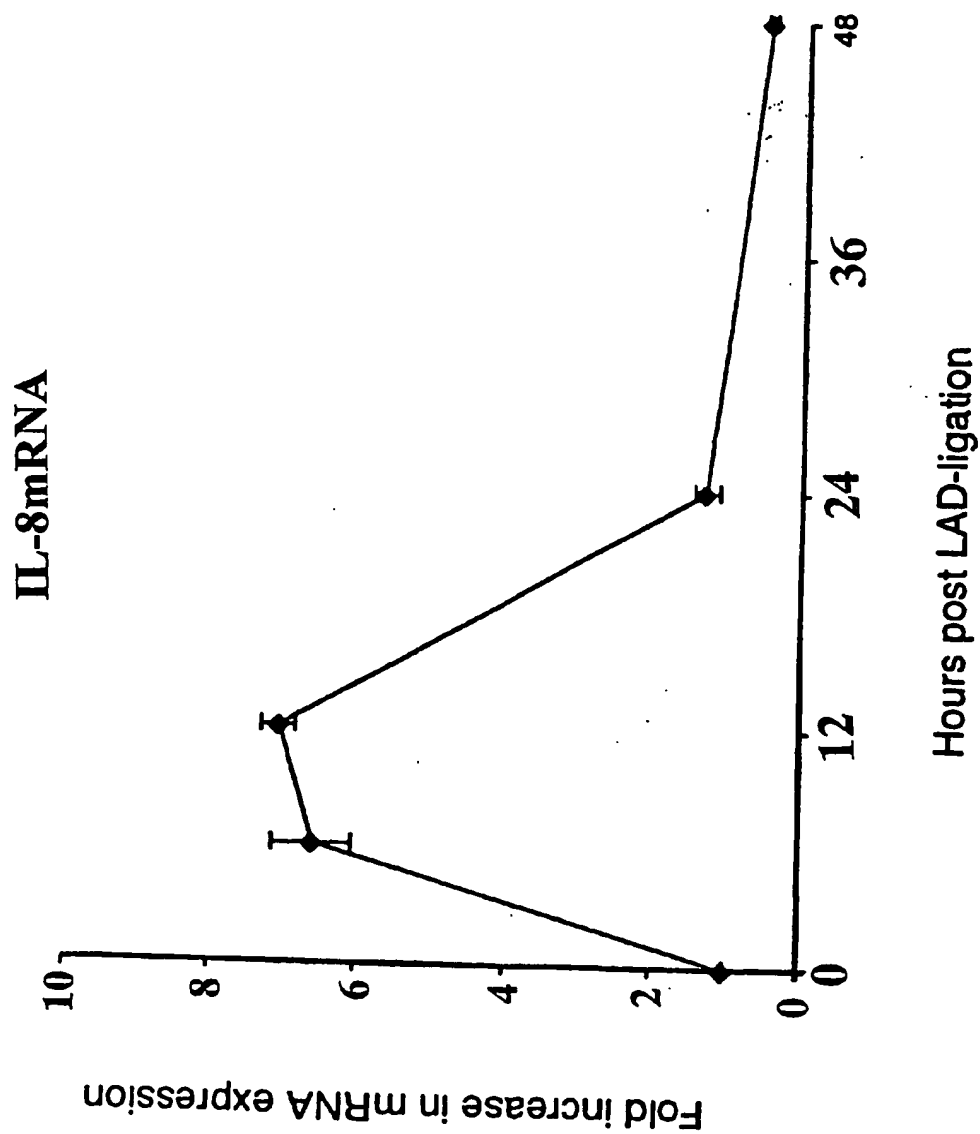
# FIGURE 7B



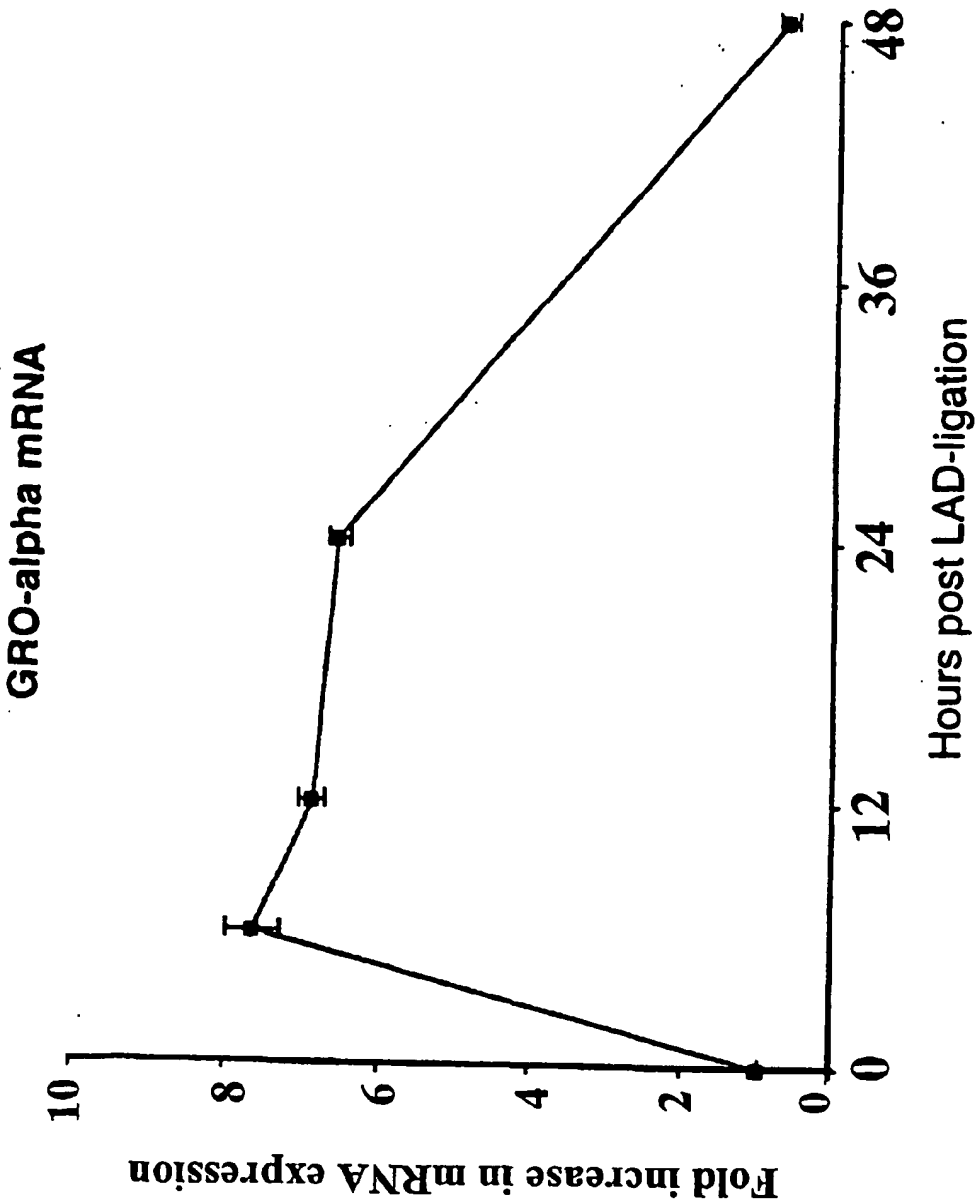
# FIGURE 7C



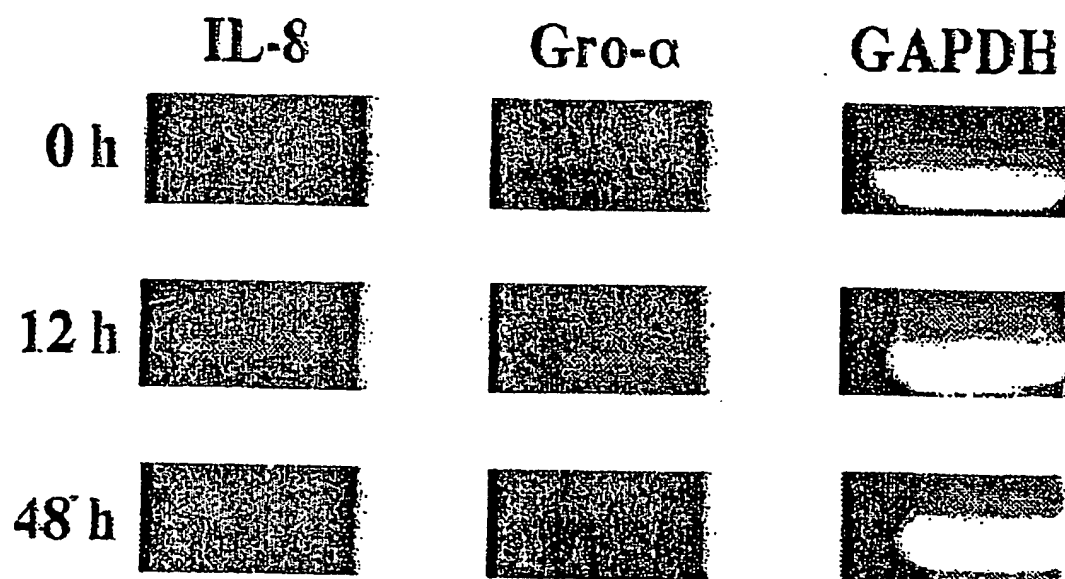
**FIGURE 8A**



**FIGURE 8B**

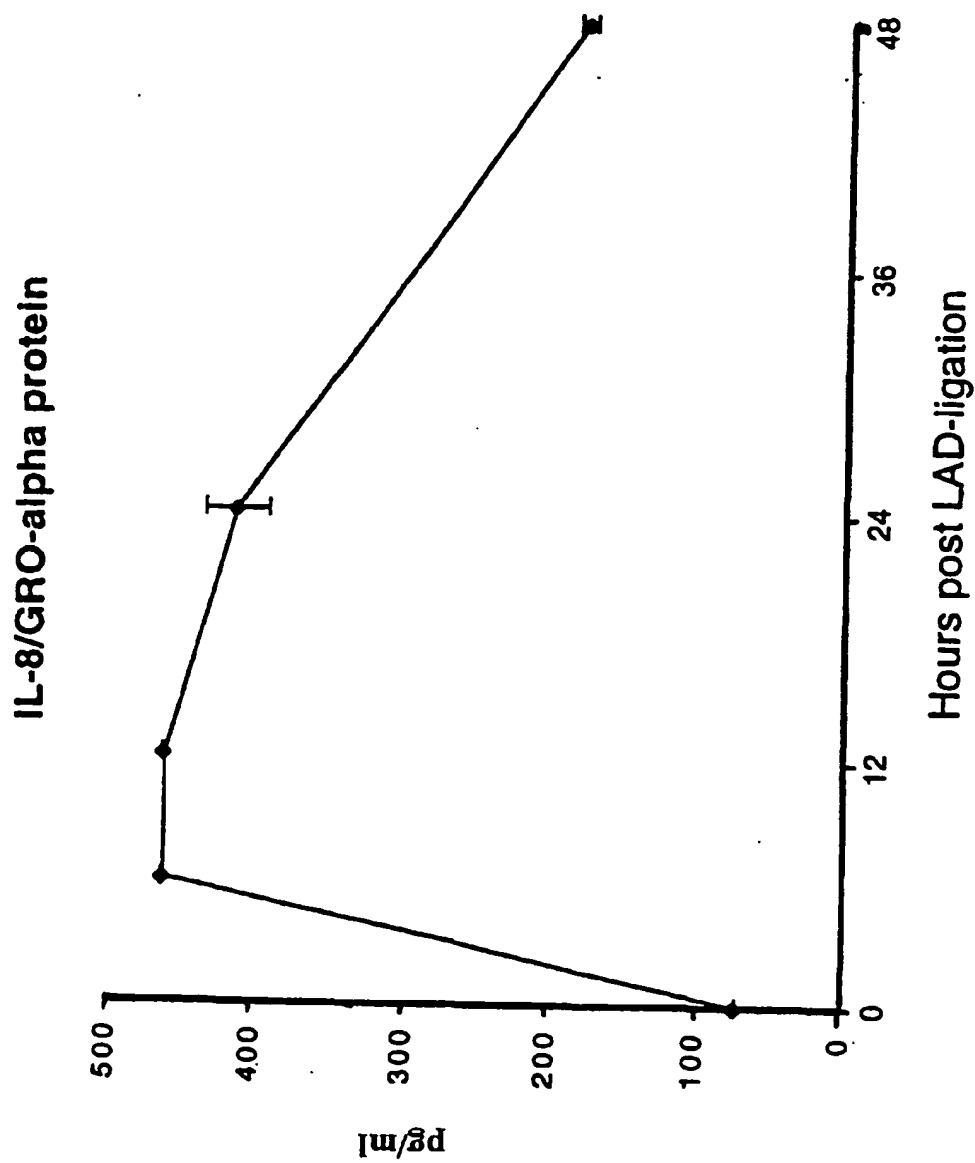


# FIGURE 8C

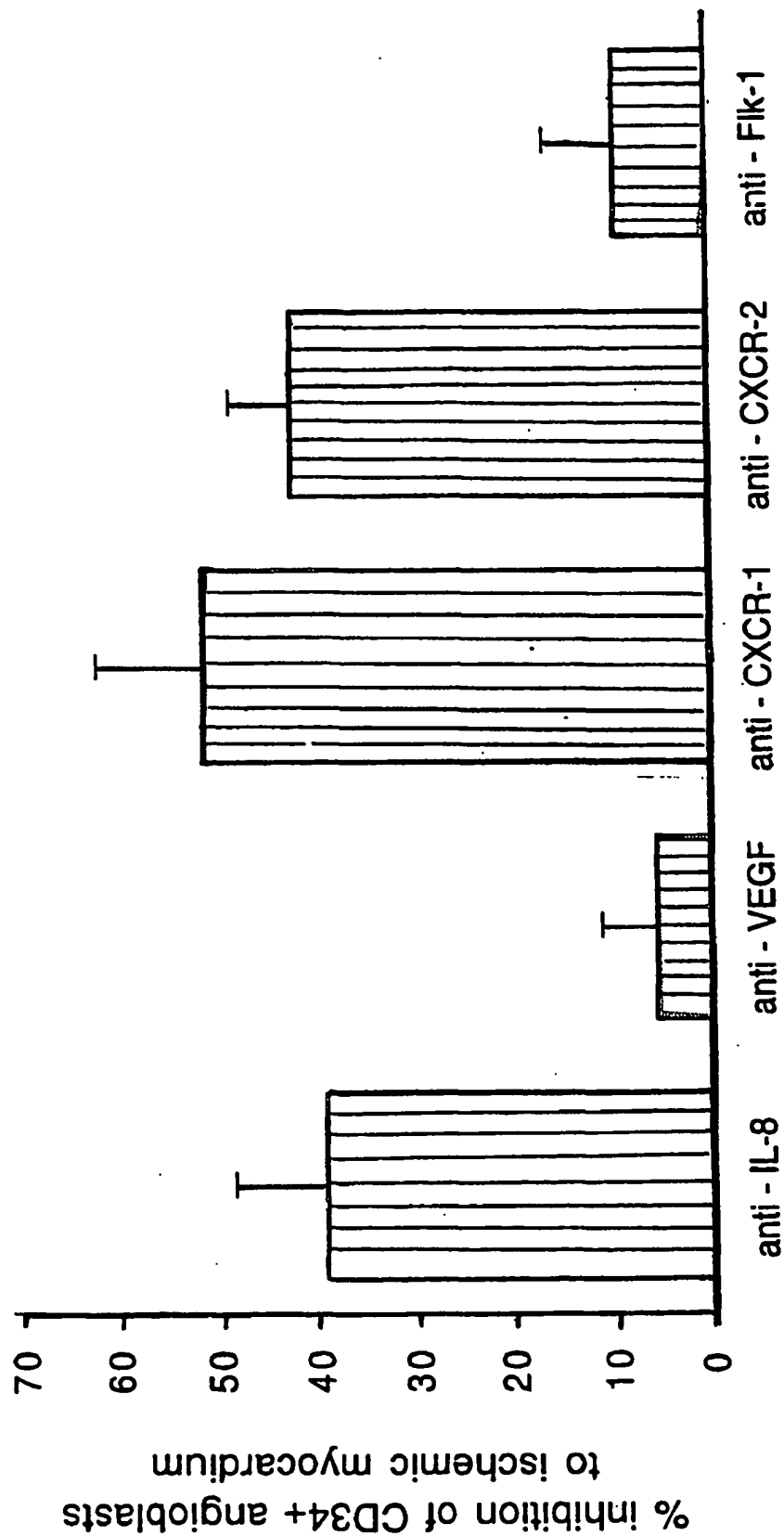




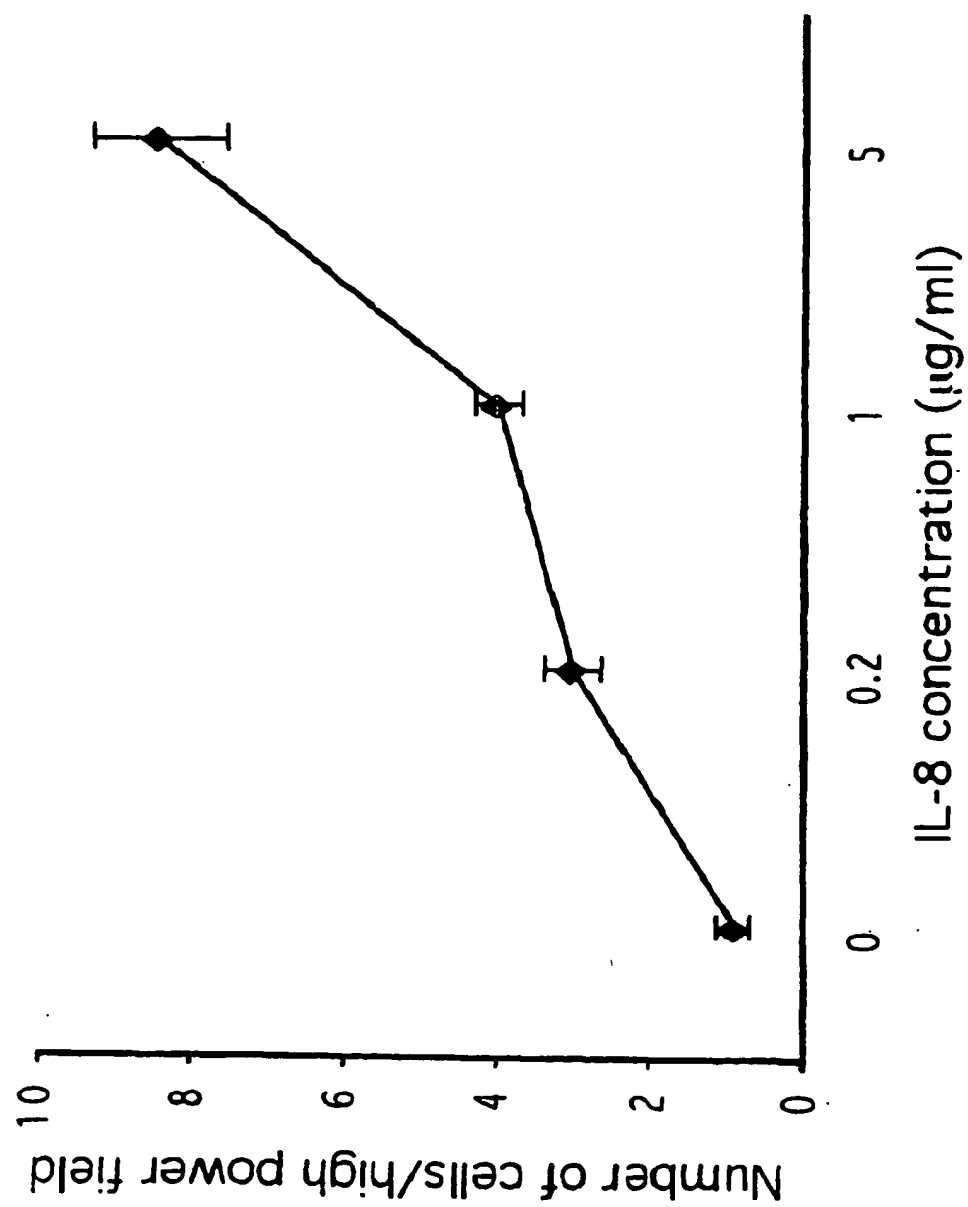
**FIGURE 8D**



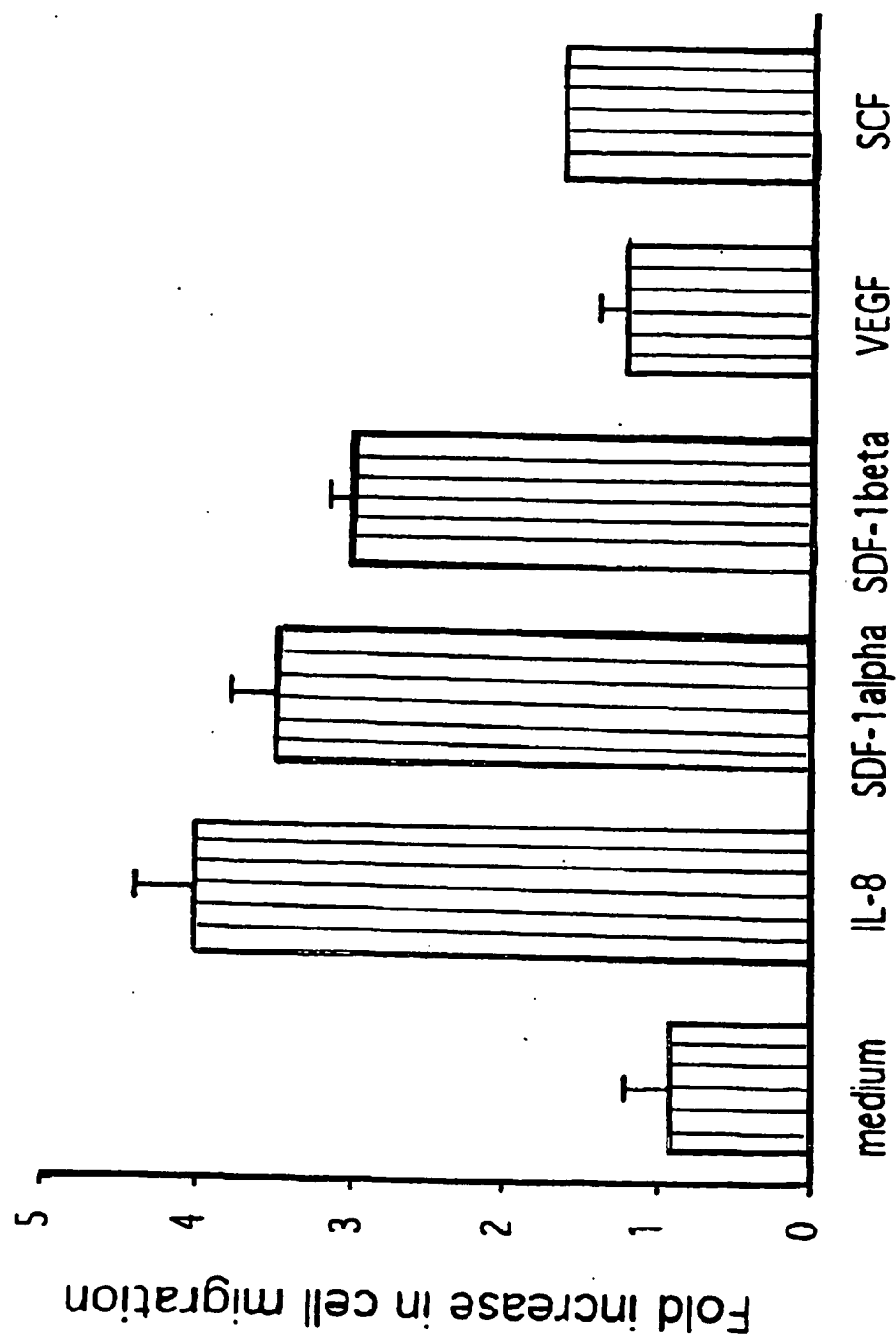
**FIGURE 8E**



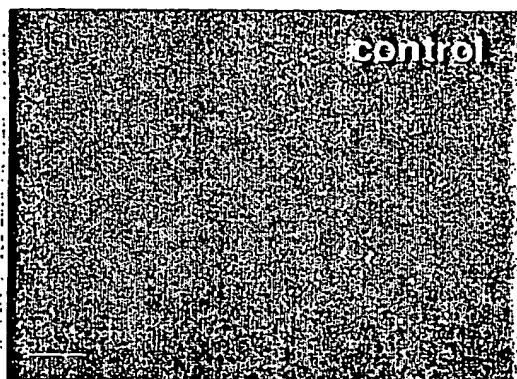
**FIGURE 9A**



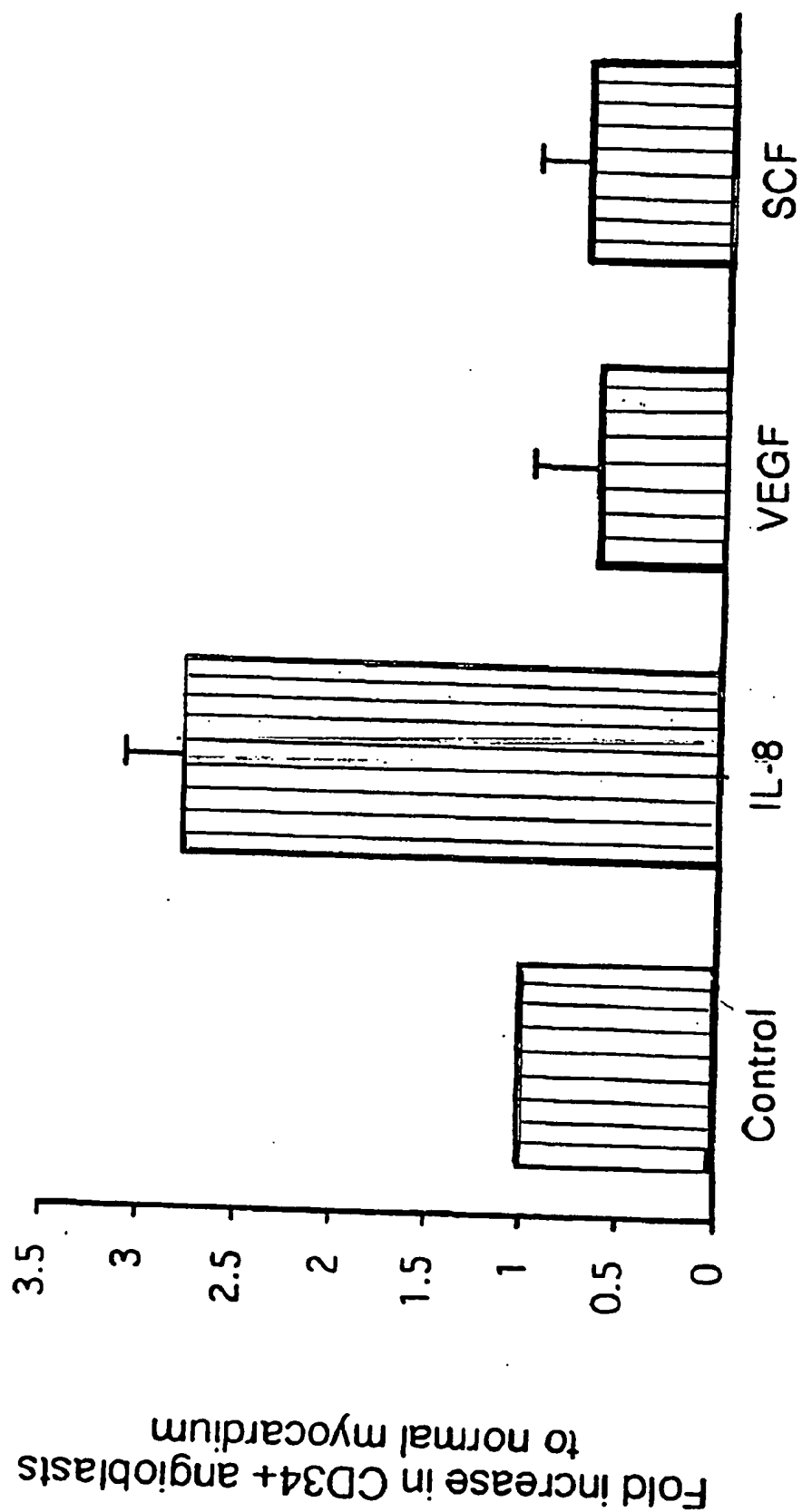
**FIGURE 9B**



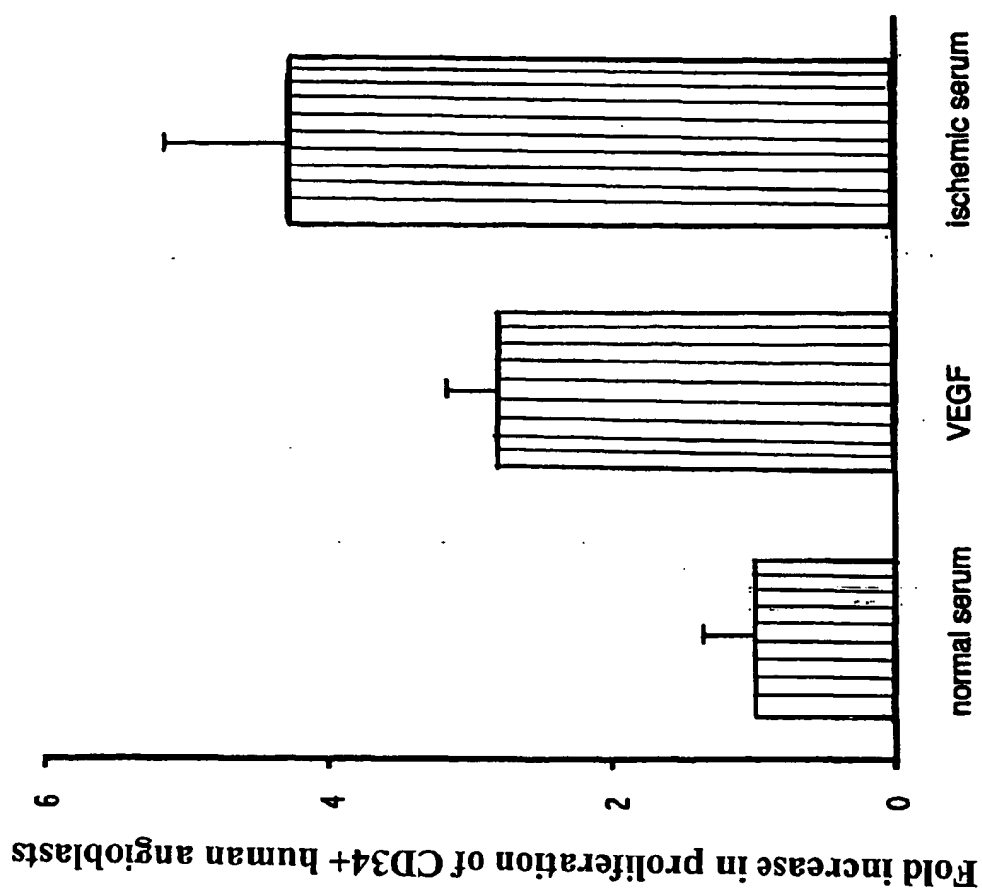
# FIGURE 9C



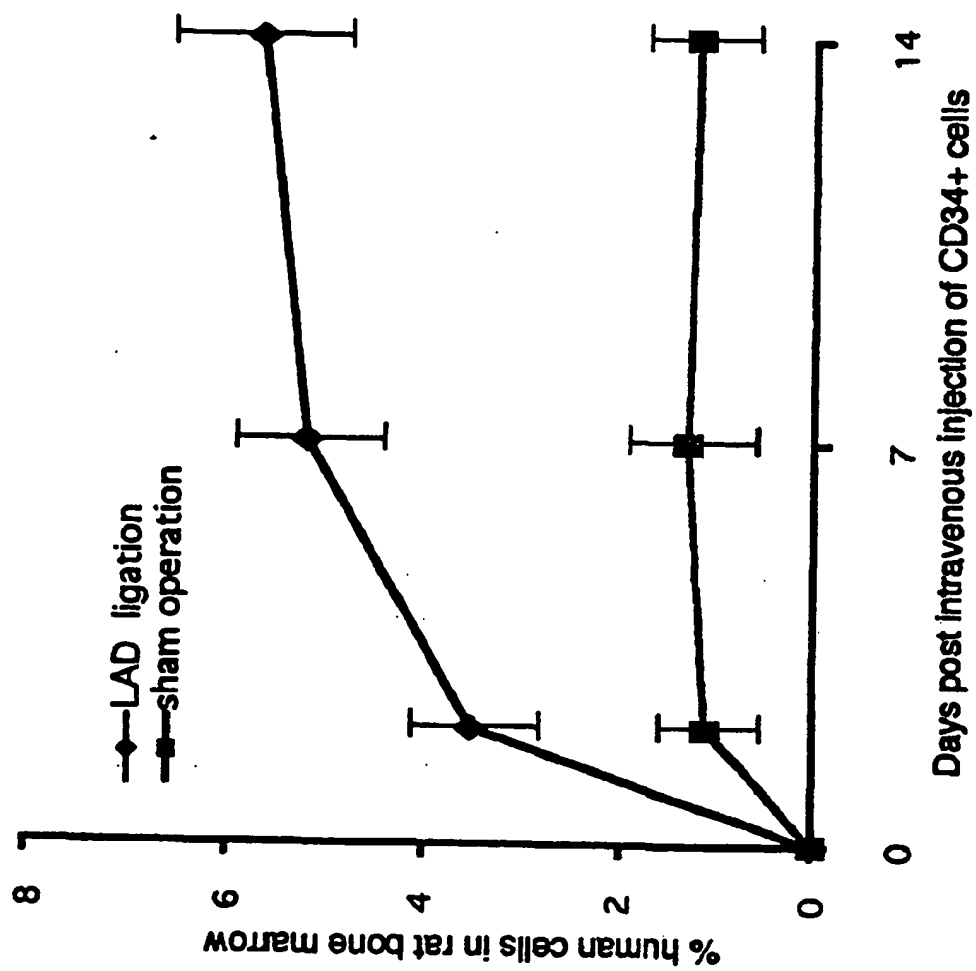
**FIGURE 9D**



**FIGURE 10A**

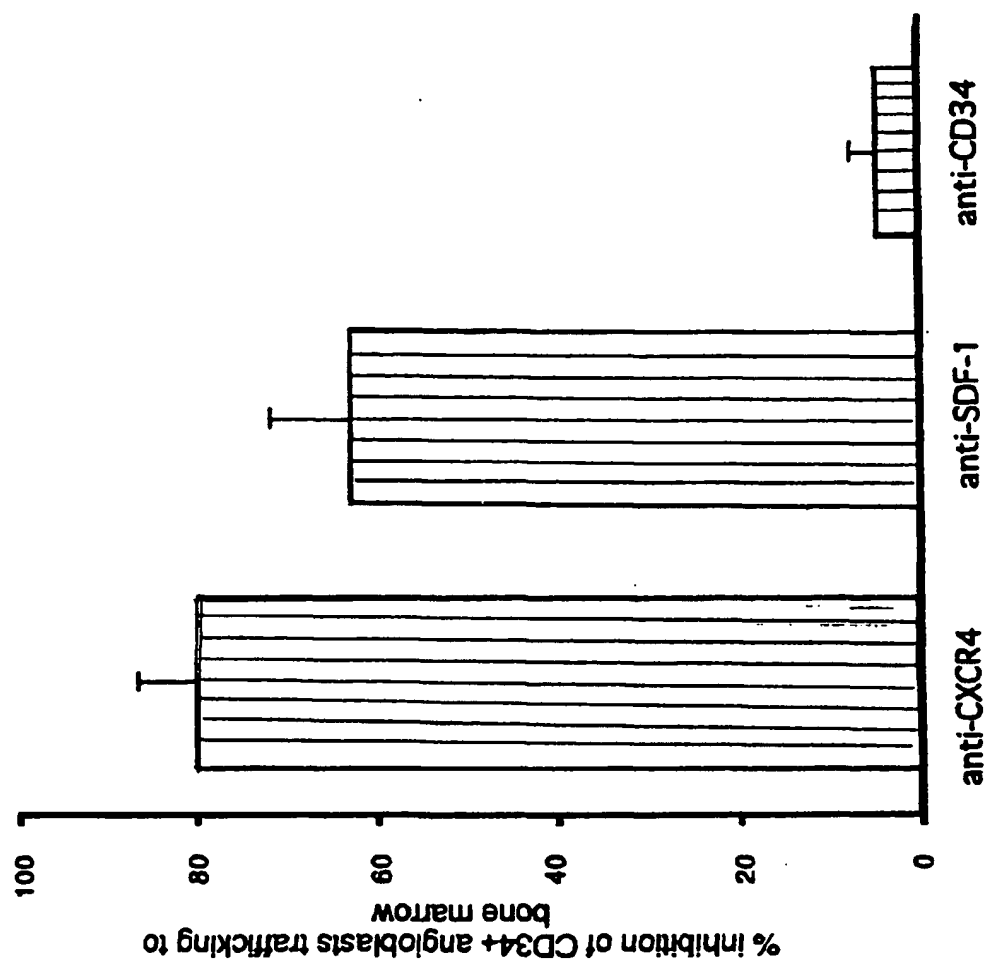


**FIGURE 10B**

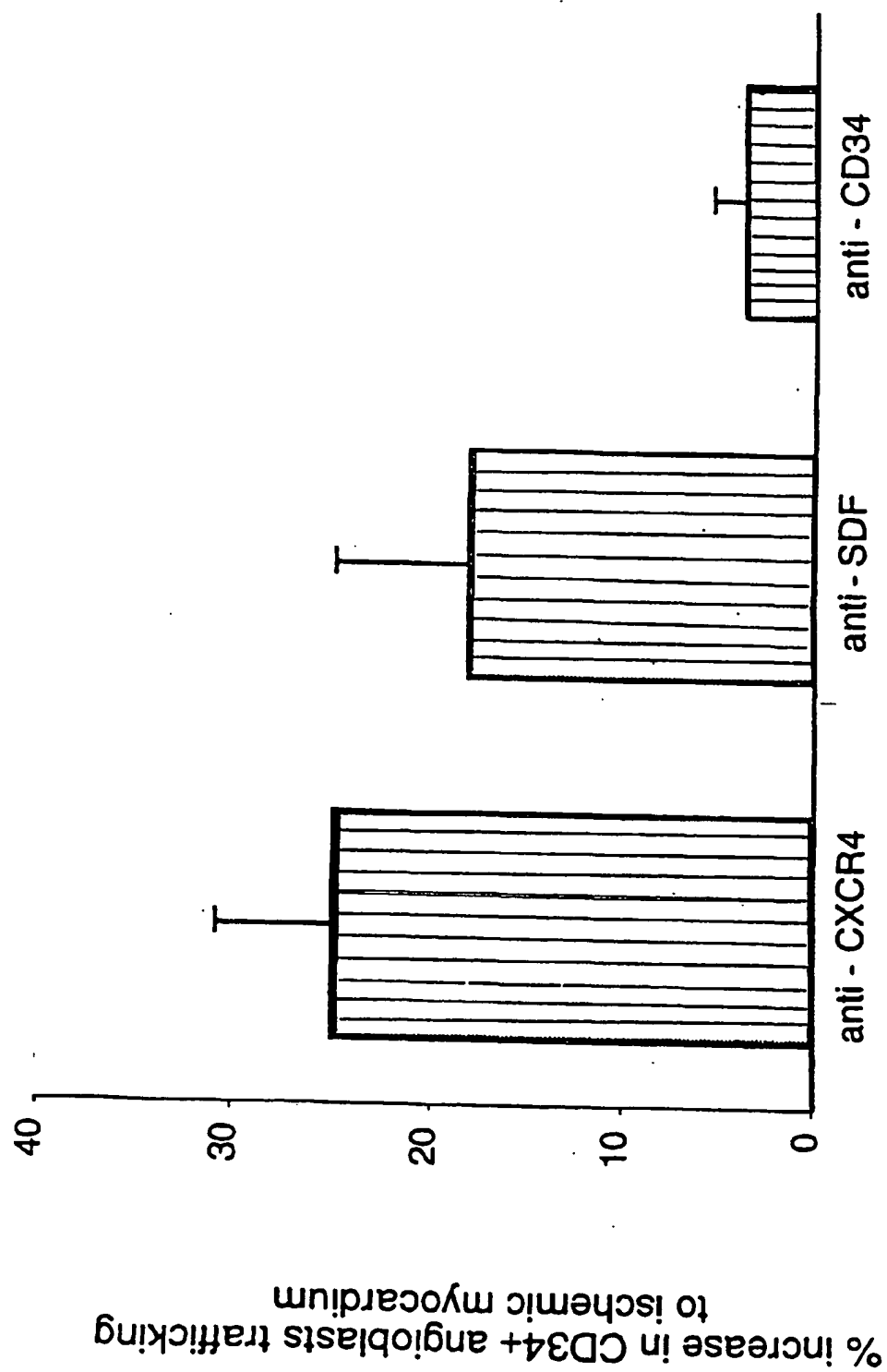




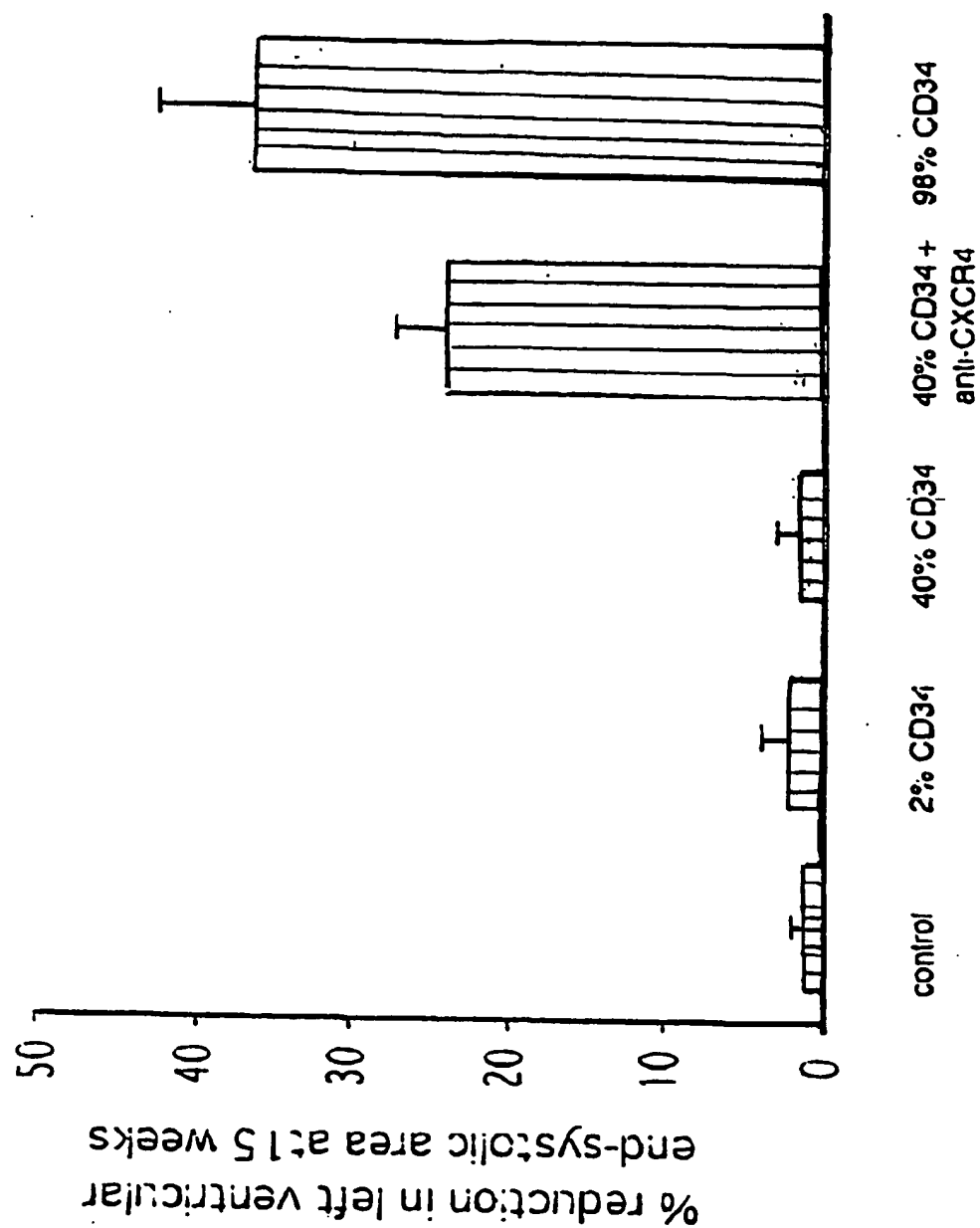
**FIGURE 10C**



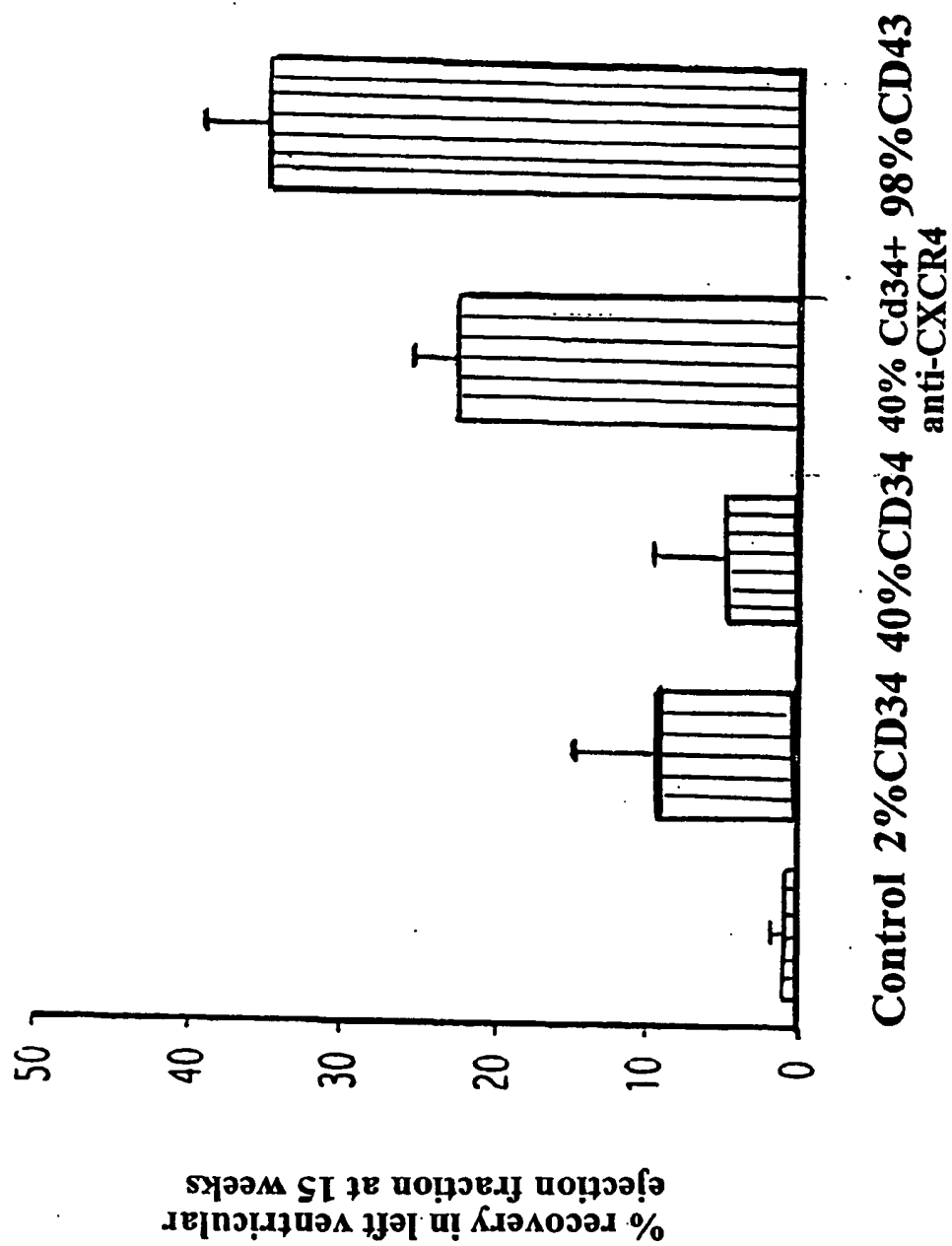
**FIGURE 10D**



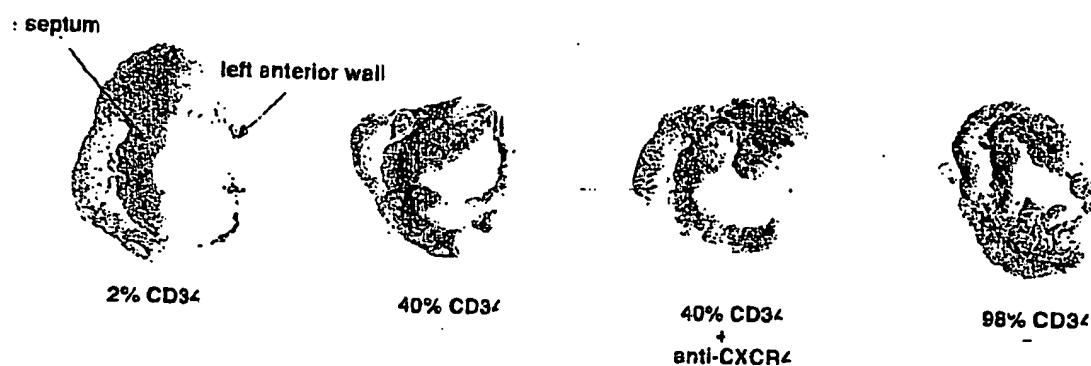
**FIGURE 11A**



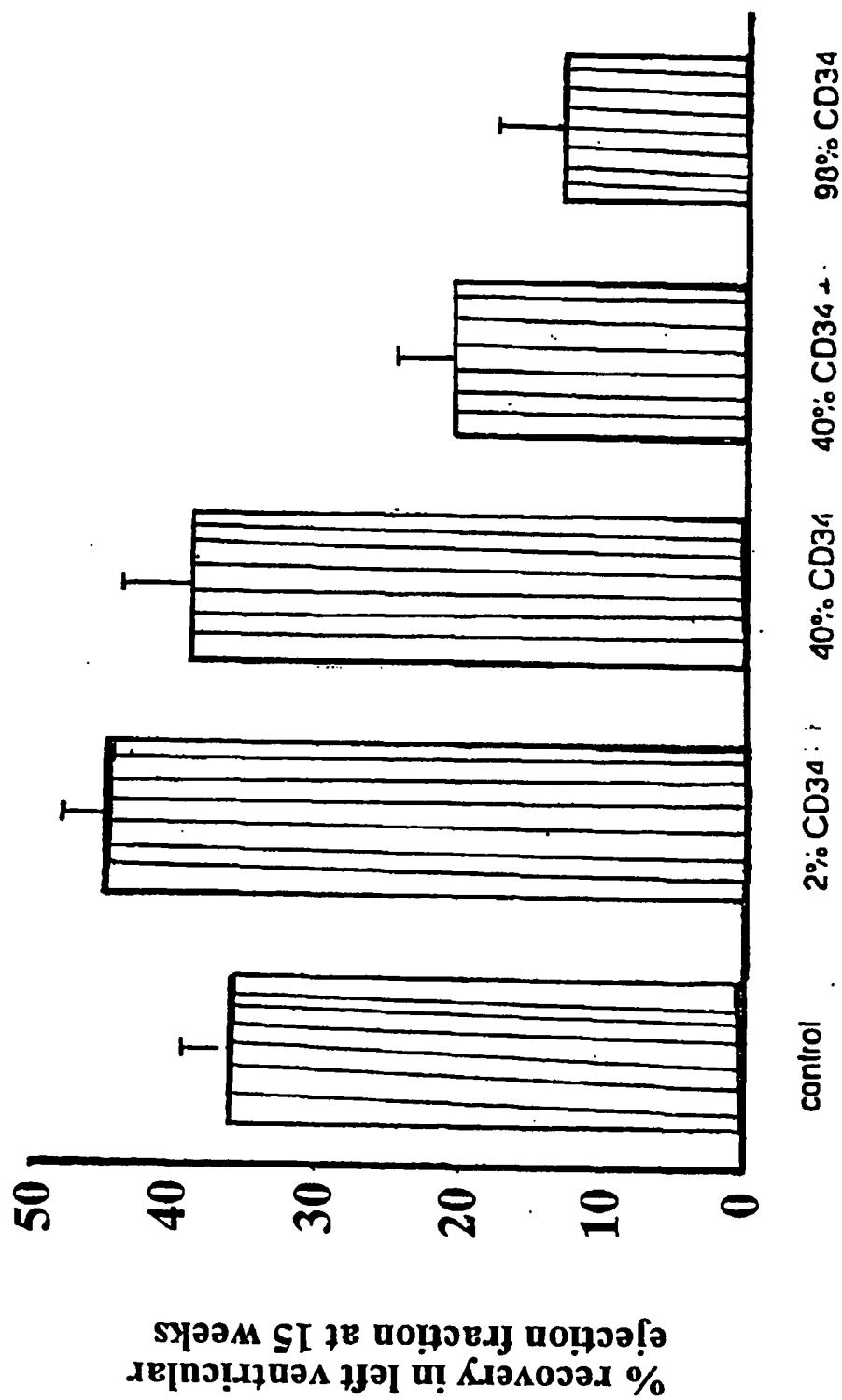
**FIGURE 11B**



# FIGURE 11C

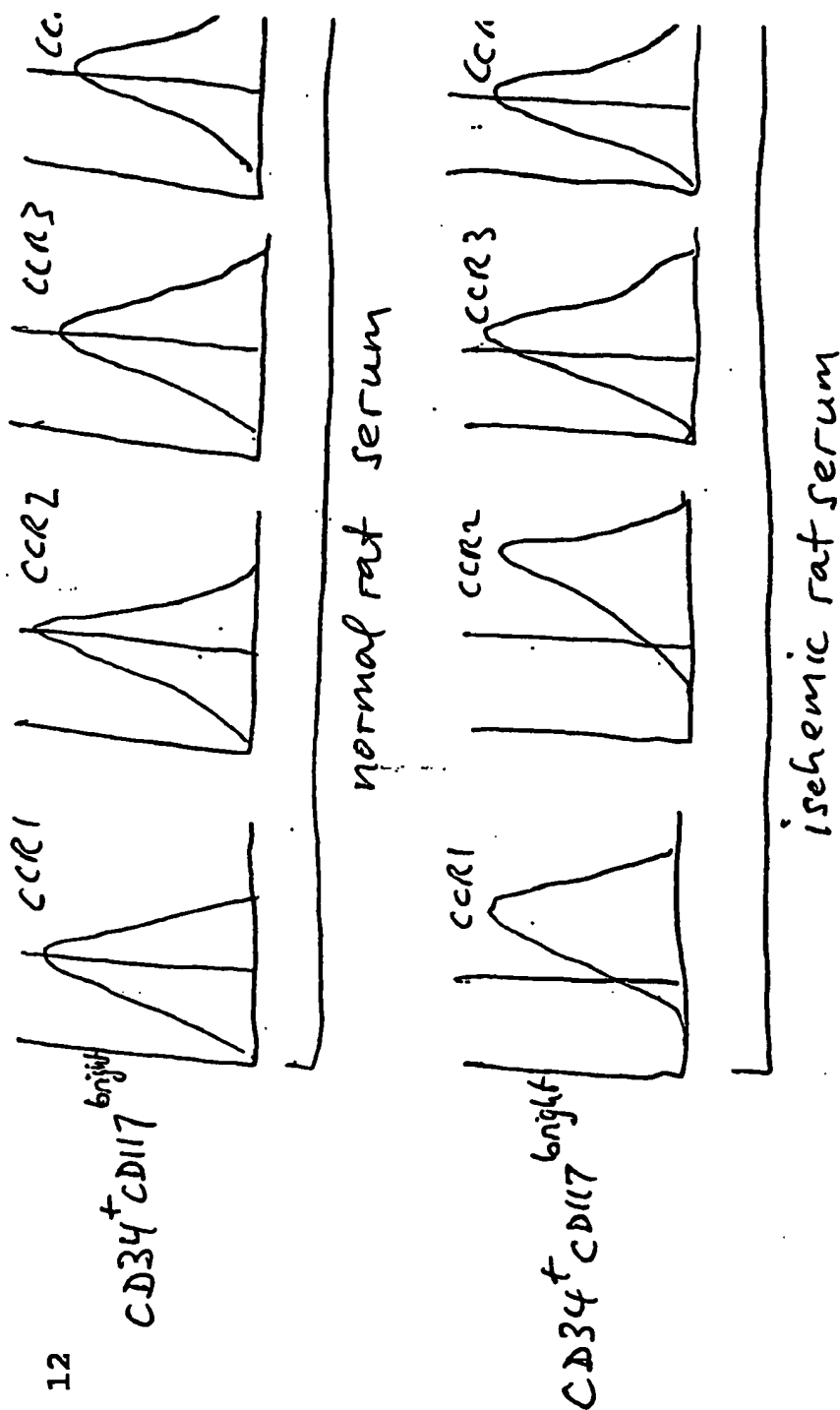


**FIGURE 11D**



# CC Chemokine Data.

Fig. 12



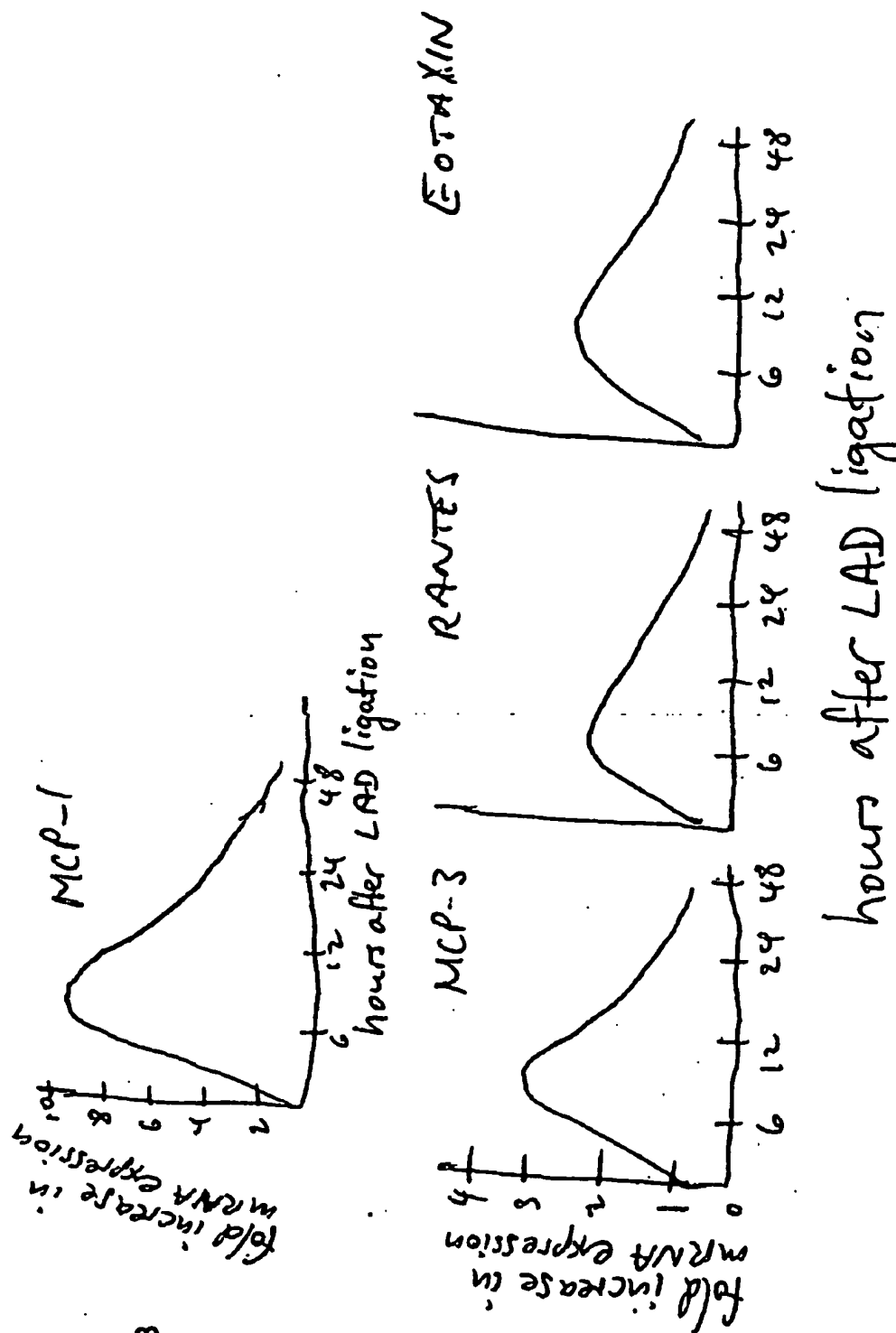


Fig. 13



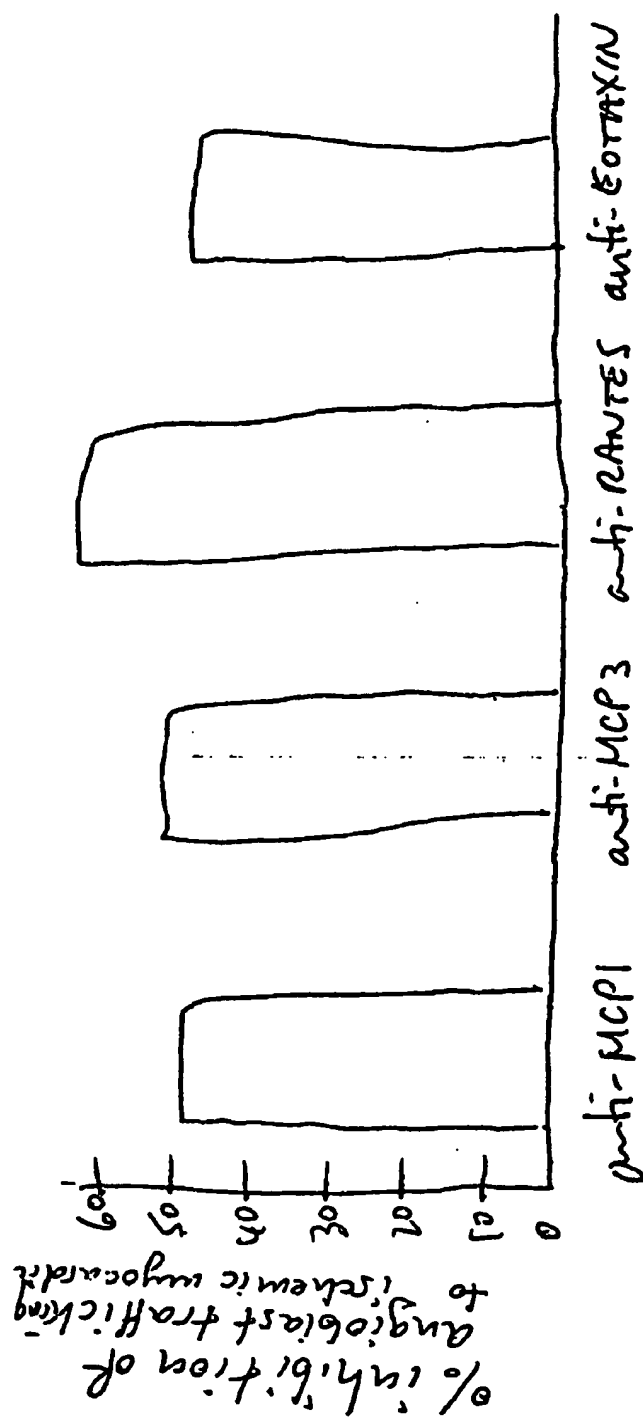


Fig. 14

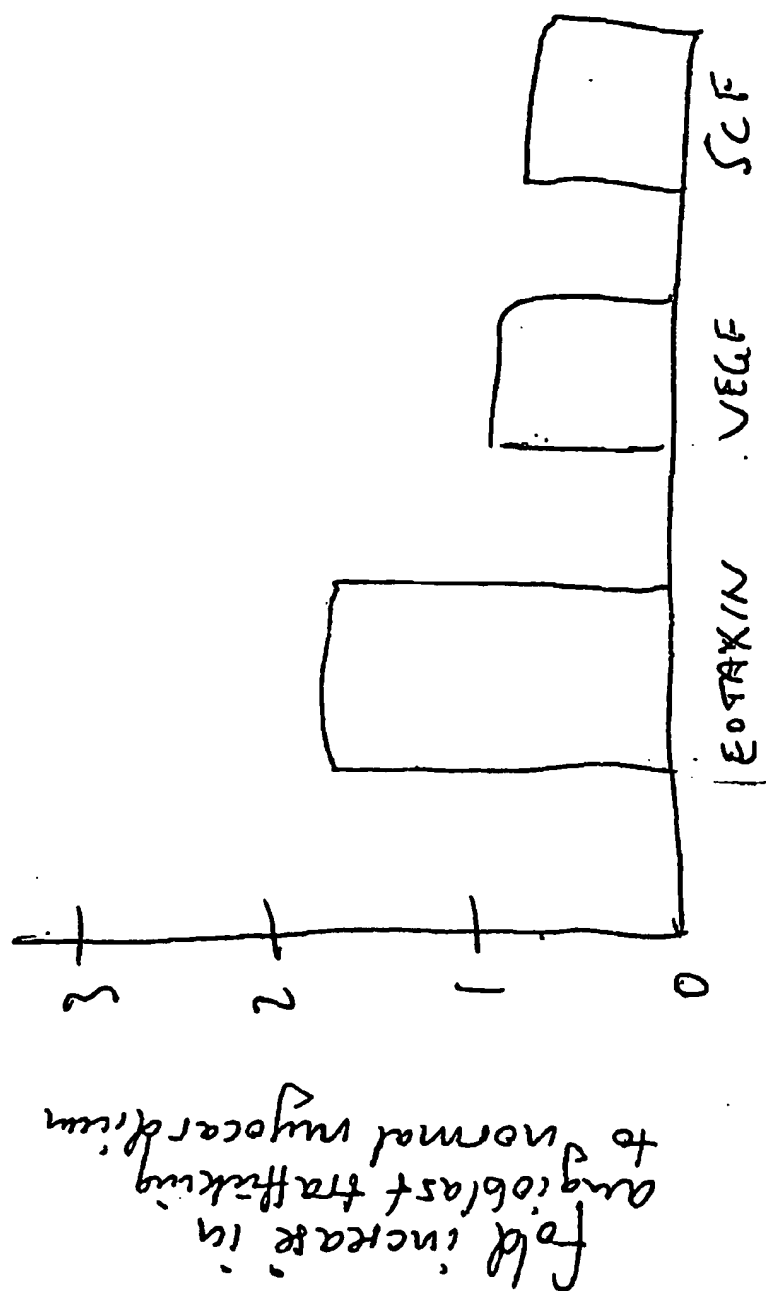


Fig. 15

**IDENTIFICATION AND USE OF HUMAN BONE  
MARROW-DERIVED ENDOTHELIAL  
PROGENITOR CELLS TO IMPROVE  
MYOCARDIAL FUNCTION AFTER ISCHEMIC  
INJURY**

[0001] This application is a continuation-in-part and claims priority of U.S. Ser. No. 09/587,441, filed Jun. 5, 2000, the contents of which are hereby incorporated by reference.

[0002] Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

**BACKGROUND OF THE INVENTION**

[0003] Left ventricular remodeling after myocardial infarction is a major cause of subsequent heart failure and death. The capillary network cannot keep pace with the greater demands of the hypertrophied but viable myocardium, resulting in myocardial death and fibrous replacement. The first series of experiments of the present invention, described below, show that human adult bone marrow contains endothelial cell precursors with phenotypic and functional characteristics of embryonic hemangioblasts, and that these can be mobilized, expanded, and used to induce infarct bed vasculogenesis after experimental myocardial infarction. The neo-angiogenesis results in significant and sustained increase in viable myocardial tissue, reduction in collagen deposition, and improved myocardial function. The use of cytokine-mobilized autologous human bone marrow-derived angioblasts for revascularization of myocardial infarct tissue, alone or in conjunction with currently used therapies, offers the potential to significantly reduce morbidity and mortality associated with left ventricular remodeling post-myocardial infarction.

[0004] Although prompt reperfusion within a narrow time window has significantly reduced early mortality from acute myocardial infarction, post-infarction heart failure is increasing and reaching epidemic proportions (1). Left ventricular remodeling after myocardial infarction, characterized by expansion of the initial infarct area, progressive thinning of the wall surrounding the infarct, and dilation of the left ventricular lumen, has been identified as a major prognostic factor for subsequent heart failure (2,3). This process is accompanied by transcription of genes normally expressed only in the fetal state, rapid and progressive increase in collagen secretion by cardiac fibroblasts, deposition of fibrous tissue in the ventricular wall, increased wall stiffness, and both diastolic and systolic dysfunction (4,5). Hypoxia directly stimulates collagen secretion by cardiac fibroblasts, while inhibiting DNA synthesis and cellular proliferation (6). In animal models, late reperfusion following experimental myocardial infarction at a point beyond myocardial salvage significantly benefits remodeling (7). Moreover, the presence of a patent infarct related artery is consistently associated with survival benefits in the post-infarction period in humans (8). This appears to be due to adequate reperfusion of the infarct vascular bed which modifies the ventricular remodeling process and prevents abnormal changes in wall motion (9).

[0005] Successful reperfusion of non-cardiac tissues rendered ischemic in experimental animal models has recently been demonstrated by use of either circulating or bone marrow-derived cellular elements (10-13). Although the precise nature of these cells was not defined in these studies, the presence of precursor cells in both adult human circulation and bone marrow which have the capability to differentiate into functional endothelial cells, a process termed vasculogenesis (14-16), has been shown. In the pre-natal period, precursor cells derived from the ventral endothelium of the aorta in human and lower species have been shown to give rise to cellular elements involved in both the processes of vasculogenesis and hematopoiesis (17,18). These cells have been termed embryonic hemangioblasts, are characterized by expression of CD34, CD117 (stem cell factor receptor), Flk-1 (vascular endothelial cell growth factor receptor-2, VEGFR-2), and Tie-2 (angiopoietin receptor), and have been shown to have high proliferative potential with blast colony formation in response to VEGF (19-22). The subsequent proliferation and differentiation of embryonic hemangioblasts to adult-type pluripotent stem cells appears to be related to co-expression of the GATA-2 transcription factor, since GATA-2 knockout embryonic stem cells have a complete block in definitive hematopoiesis and seeding of the fetal liver and bone marrow (23). Moreover, the earliest precursor of both hematopoietic and endothelial cell lineage to have diverged from embryonic ventral endothelium has been shown to express VEGF receptors as well as GATA-2 and alpha4-integrins (24). The first series of experiments of the present invention shows that GATA-2 positive stem cell precursors are also present in adult human bone marrow, demonstrate properties of hemangioblasts, and can be used to induce vasculogenesis, thus preventing remodeling and heart failure in experimental myocardial infarction.

[0006] Growth of new vessels from pre-existing mature endothelium has been termed angiogenesis, and can be regulated by many factors including certain CXC chemokines (47-50). In contrast, vasculogenesis is mediated by bone marrow-derived endothelial precursors (51-53) with phenotypic characteristics of embryonic angioblasts and growth/differentiation properties regulated by receptor tyrosine kinases such as vascular endothelial growth factor (VEGF) (54-57). Therapeutic vasculogenesis (58-61) has the potential to improve perfusion of ischemic tissues, however the receptor/ligand interactions involved in selective trafficking of endothelial precursors to sites of tissue ischemia are not known. The second series of experiments of the present invention, described below, show that vasculogenesis can develop in infarcted myocardium as a result of interactions between CXC receptors on human bone marrow-derived angioblasts and ELR-positive CXC chemokines induced by ischemia, including IL-8 and Gro-alpha. Moreover, redirected trafficking of angioblasts from the bone marrow to ischemic myocardium can be achieved by blocking CXCR4/SDF-1 interactions, resulting in increased vasculogenesis, decreased myocardial death and fibrous replacement, and improved cardiac function. The results of the experiments indicate that CXC chemokines, including IL-8, Gro-alpha, and stromal-derived factor-1 (SDF-1), play a central role in regulating vasculogenesis in the adult human, and suggest that manipulating interactions between CXC chemokines and their receptors on bone marrow-derived angioblasts can lead to optimal therapeutic vasculogenesis and salvage of

ischemic tissues. The third series of experiments, described below, show that CC chemokines also play a role in mediating angioblast chemotaxis to ischemic myocardium.

[0007] The angiogenic response during wound repair or inflammation is thought to result from changes in adhesive interactions between endothelial cells in pre-existing vasculature and extracellular matrix which are regulated by locally-produced factors and which lead to endothelial cell migration, proliferation, reorganization and microvessel formation (70). The human CXC chemokine family consists of small (<10 kD) heparin-binding polypeptides that bind to and have potent chemotactic activity for endothelial cells. Three amino acid residues at the N-terminus (Glu-Leu-Arg, the ELR motif) determine binding of CXC chemokines such as IL-8 and Gro-alpha to CXC receptors 1 and 2 on endothelial cells (49,71), thus promoting endothelial chemotaxis and angiogenesis (47-48). In contrast, CXC chemokines lacking the ELR motif bind to different CXC receptors and inhibit growth-factor mediated angiogenesis (49-72). Although SDF-1, an ELR-negative CXC chemokine, is a potent inducer of endothelial chemotaxis through interactions with CXCR4 (73), its angiogenic effects appear to be limited to the developing gastrointestinal tract vascular system (50).

[0008] Vasculogenesis first occurs during the pre-natal period, with haemangioblasts derived from the human ventral aorta giving rise to both endothelial and haematopoietic cellular elements (74,75). Similar endothelial progenitor cells have recently been identified in adult human bone marrow (51-53), and shown to have the potential to induce vasculogenesis in ischemic tissues (59-61). However, the signals from ischemic sites required for chemoattraction of such bone marrow-derived precursors, and the receptors used by these cells for selective trafficking to these sites, are unknown. Following myocardial infarction a process of neoangiogenesis occurs (62,63), but is insufficient to sustain viable tissue undergoing compensatory hypertrophy, leading to further cell death, expansion of the initial infarct area, and collagen replacement (64-66). This process, termed remodeling, results in progressive heart failure (67-69). In the experiments described below, a nude rat model of myocardial infarction was used to investigate whether CXC chemokines containing the ELR motif regulate migration of human bone marrow-derived angioblasts to sites of tissue ischemia. Moreover, since selective bone marrow homing and engraftment of haematopoietic progenitors depends on CXCR4 binding to SDF-1 expressed constitutively in the bone marrow (76-78), whether interruption of CXCR4/SDF-1 interactions could redirect trafficking of human bone marrow-derived angioblasts to sites of tissue ischemia, thereby augmenting therapeutic vasculogenesis, was examined. The results of the experiments indicate that CXC chemokines, including IL-8, Gro-alpha, and SDF-1, play a central role in regulating human adult bone marrow-dependent vasculogenesis. Further, the fourth series of experiments described below show that stem cells can induce angiogenesis in peri-infarct tissue.

#### SUGARY OF THE INVENTION

[0009] This invention provides a method of stimulating vasculogenesis in ischemia-damaged tissue of a subject comprising:

[0010] (a) removing stem cells from a location within the subject;

[0011] (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and

[0012] (c) introducing the endothelial progenitor cells from step (b) into a different location within the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.

[0013] This invention also provides the instant method, wherein subsequent to step (b), but before step (c), the endothelial progenitor cells are expanded by contacting them with a growth factor.

[0014] This invention also provides the instant method, wherein the growth factor is a cytokine.

[0015] This invention also provides the instant method, wherein the cytokine is VEGF, FGF, G-CSF, IGF, M-CSF, or GM-CSF.

[0016] This invention also provides the instant method, wherein the growth factor is a chemokine.

[0017] This invention also provides the instant method, wherein the chemokine is Interleukin-8.

[0018] This invention also provides the instant method, wherein the endothelial progenitor cells are separated from other stem cells before expansion.

[0019] This invention also provides the instant method, wherein the ischemia-damaged tissue is myocardium.

[0020] This invention also provides the instant method, wherein the ischemia-damaged tissue is nervous system tissue.

[0021] This invention also provides the instant method, wherein the stem cells are removed from the subject's bone marrow.

[0022] This invention also provides the instant method, wherein the removal of the stem cells from the bone marrow is effected by aspiration from the subject's bone marrow.

[0023] This invention also provides the instant method, wherein the removal of the stem cells from the subject is effected by a method comprising:

[0024] (a) introducing a growth factor into the subject to mobilize the stem cells into the subject's blood; and

[0025] (b) removing a sample of blood containing the stem cells from the subject.

[0026] This invention also provides the instant method, wherein the growth factor is introduced into the subject subcutaneously, orally, intravenously or intramuscularly.

[0027] This invention also provides the instant method, wherein the growth factor is a chemokine that induces mobilization.

[0028] This invention also provides the instant method, wherein the chemokine is Interleukin-8.

[0029] This invention also provides the instant method, wherein the growth factor is a cytokine.

[0030] This invention also provides the instant method, wherein the cytokine is G-CSF, M-CSF, or GM-CSF.

[0031] This invention also provides the instant method, wherein the endothelial progenitor cells are recovered based upon their expression of CD117.

[0032] This invention also provides the instant method, wherein the endothelial progenitor cells are recovered based upon their expression of a GATA-2 activated gene product.

[0033] This invention also provides the instant method, wherein the endothelial progenitor cells are recovered based upon their expression of one or more of CD34, VEGF-R, Tie-2, GATA-3 or AC133.

[0034] This invention also provides the instant method, wherein the subject has suffered or is suffering from one or more of the following: myocardial infarction, chronic heart failure, ischemic heart disease, coronary artery disease, diabetic heart disease, hemorrhagic stroke, thrombotic stroke, embolic stroke, limb ischemia, or another disease in which tissue is rendered ischemic.

[0035] This invention also provides the instant method, wherein step (a) occurs prior to the subject suffering ischemia-damaged tissue and wherein step (c) occurs after the subject has suffered ischemia-damaged tissue.

[0036] This invention also provides the instant method, wherein the endothelial progenitor cells are frozen for a period of time between steps (b) and (c).

[0037] This invention also provides the instant method, wherein the endothelial progenitor cells are frozen for a period of time after being expanded but before step (c) is performed.

[0038] This invention also provides the instant method, wherein the endothelial progenitor cells are introduced into the subject by injection directly into the peripheral circulation, heart muscle, left ventricle, right ventricle, coronary artery, cerebro-spinal fluid, neural tissue, ischemic tissue, or post-ischemic tissue.

[0039] This invention also provides the instant method, further comprising administering to the subject one or more of the following: an inhibitor of Plasminogen Activator Inhibitor, Angiotensin Converting Enzyme Inhibitor or a beta blocker, wherein such administration occurs prior to, concomitant with, or following step (c).

[0040] This invention also provides a method of stimulating angiogenesis in peri-infarct tissue in a subject comprising:

[0041] (a) removing stem cells from a location within a subject;

[0042] (b) recovering endothelial progenitor cells from the stem cells removed in step (a);

[0043] (c) expanding the endothelial progenitor cells recovered in step (b) by contacting the progenitor cells with a growth factor; and

[0044] (d) introducing the expanded endothelial progenitor cells from step (c) into a different location in the subject such that the endothelial progenitor cells stimulate angiogenesis in peri-infarct tissue in the subject.

[0045] This invention also provides a method of selectively increasing the trafficking of endothelial progenitor cells to ischemia-damaged tissue in a subject comprising:

[0046] (a) administering endothelial progenitor cells to a subject; and

[0047] (b) administering a chemokine to the subject so as to thereby attract the endothelial progenitor cells to the ischemia-damaged tissue.

[0048] This invention also provides the instant method, wherein the chemokine is administered to the subject prior to administering the endothelial progenitor cells.

[0049] This invention also provides the instant method, wherein the chemokine is administered to the subject concurrently with the endothelial progenitor cells.

[0050] This invention also provides the instant method, wherein the chemokine is administered to the subject after administering the endothelial progenitor cells.

[0051] This invention also provides the instant method, wherein the chemokine is a CXC chemokine.

[0052] This invention also provides the instant method, wherein the CXC chemokine is selected from the group consisting of Interleukin-8, Gro-Alpha, or Stromal-Derived Factor-1.

[0053] This invention also provides the instant method, wherein the chemokine is a CC chemokine.

[0054] The method of claim 34, wherein the CC chemokine is selected from the group consisting of RANTES, EOTAXIN, MCP-1, MCP-2, MCP-3, or MCP-4.

[0055] This invention also provides the instant method, wherein the chemokine is administered to the subject by injection into the subject's peripheral circulation, heart muscle, left ventricle, right ventricle, coronary arteries, cerebro-spinal fluid, neural tissue, ischemic tissue, or post-ischemic tissue.

[0056] This invention also provides a method of increasing trafficking of endothelial progenitor cells to ischemia-damaged tissue in a subject comprising inhibiting any interaction between Stromal-Derived Factor-1 and CXCR4.

[0057] This invention also provides the instant method, wherein interaction between Stromal-Derived Factor-1 (SDF-1) and CXCR4 is inhibited by administration of an anti-SDF-1 or an anti-CXCR4 monoclonal antibody to the subject.

[0058] This invention also provides the instant method, further comprising administering to the subject an angiotensin converting enzyme inhibitor, an AT<sub>1</sub>-receptor blocker, or a beta blocker.

[0059] This invention also provides a method of reducing trafficking of endothelial progenitor cells to bone marrow in a subject comprising inhibiting production of Stromal-Derived Factor-1 in the subject's bone marrow.

[0060] This invention also provides the instant method, wherein SDF-1 production is inhibited by administration of an anti-SDF-1 or anti-CXCR4 monoclonal antibody to the subject.

[0061] This invention also provides a method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific chemokine produced by proliferating cells associ-

ated with the cancer so as to reduce trafficking of endothelial progenitor cells to such proliferating cells and thereby treat the cancer in the subject.

[0062] This invention also provides a method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific receptor located on an endothelial progenitor cell, for a chemokine produced by proliferating cells associated with the cancer, so as to reduce trafficking of the endothelial progenitor cell to such proliferating cells and thereby treat the cancer in the subject.

[0063] This invention also provides a method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce vasculogenesis in the subject's tumor and thereby treat the tumor.

[0064] This invention also provides a method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce angiogenesis in the subject's tumor and thereby treat the tumor.

[0065] This invention also provides the instant method, wherein the receptor is a CD117 receptor.

[0066] This invention also provides a method for expressing a gene of interest in an endothelial progenitor cell or a mast progenitor cell which comprises inserting into the cell a vector comprising a promoter containing a GATA-2 motif and the gene of interest.

[0067] This invention also provides the instant method, wherein the vector is inserted into the cell by transfection.

[0068] This invention also provides the instant method, wherein the promoter is a preproendothelin-1 promoter.

[0069] This invention also provides the instant method, wherein the promoter is of mammalian origin.

[0070] This invention also provides the instant method, wherein the promoter is of human origin.

[0071] This invention provides a composition comprising an amount of a monoclonal antibody directed against an epitope of a specific chemokine produced by a cancer effective to reduce trafficking of endothelial progenitor cells to the cancer, and a pharmaceutically acceptable carrier.

[0072] This invention provides a method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

[0073] (a) removing stem cells from a location within the subject;

[0074] (b) recovering endothelial progenitor cells from the stem cells removed in step (a);

[0075] (c) recovering those endothelial progenitor cells recovered in step (b) that express GATA-2;

[0076] (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and

[0077] (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality.

[0078] This invention provides a method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

[0079] (a) removing stem cells from a location within the subject;

[0080] (b) recovering mast progenitor cells from the stem cells removed in step (a);

[0081] (c) recovering those mast progenitor cells recovered in step (b) that express GATA-2;

[0082] (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and

[0083] (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality

[0084] This invention provides the instant method, wherein the abnormality is ischemia-damaged tissue.

[0085] This invention provides the instant method, wherein the gene product is proendothelin.

[0086] This invention provides the instant method, wherein the gene product is endothelin.

[0087] This invention provides the a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising:

[0088] (a) removing stem cells from a location in the subject;

[0089] (b) recovering cells that express CD117 from the stem cells; and

[0090] (c) introducing the recovered cells into a different location in the subject such that the cells improve myocardial function in the subject.

[0091] This invention provides the instant methods, wherein the subject is of mammalian origin.

[0092] This invention provides the instant method, wherein the mammal is of human origin.

[0093] This invention also provides a method of stimulating vasculogenesis in ischemia-damaged tissue in a subject comprising:

[0094] (a) obtaining allogeneic stem cells;

[0095] (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and

[0096] (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.

[0097] This invention provides the instant method, wherein the allogeneic stem cells are obtained from embryonic, fetal or cord blood sources.

[0098] This invention provides a method of stimulating angiogenesis in ischemia-damaged tissue in a subject comprising:

[0099] (a) obtaining allogeneic stem cells;

[0100] (b) recovering endothelial progenitor cells in the stem cells removed in step (a); and

[0101] (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate angiogenesis in the subject's ischemia-damaged tissue.

[0102] This invention provides the instant method, wherein the allogeneic stem cells are obtained from embryonic, fetal or cord blood sources.

[0103] This invention also provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting G-CSF into the subject in order to mobilize endothelial progenitor cells.

[0104] This invention also provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting anti-CXCR4 antibody into the subject.

[0105] This invention also provides the instant method further comprising introducing endothelial progenitor cells into the subject.

[0106] This invention also provides the instant method further comprising introducing G-CSF into the subject in order to mobilize endothelial progenitor cells.

#### BRIEF DESCRIPTION OF THE FIGURES

[0107] FIG. 1. G-CSF Mobilizes Two Human Bone Marrow-Derived Populations Expressing VEGF Receptors: One With Characteristics Of Mature Endothelial Cells. And A Second With Characteristics Of Embryonic Angioblasts.

[0108] A-D depicts four-parameter flow cytometric phenotype characterization of G-CSF mobilized bone marrow-derived cells removed by leukapheresis from a representative human donor adult (25). Only live cells were analyzed, as defined by 7-AAD staining. For each marker used, shaded areas represent background log fluorescence relative to isotype control antibody.

[0109] A. Following immunoselection of mononuclear cells (25), >95% of live cells express CD34.

[0110] B. The CD34+CD117<sup>dim</sup> subset contains a population with phenotypic characteristics of mature, vascular endothelium.

[0111] C. The CD34+CD117<sup>bright</sup> subset contains a population expressing markers characteristic of primitive hemangioblasts arising during waves of murine and human embryogenesis.

[0112] D. CD34+CD117<sup>bright</sup> cells co-expressing GATA-2 and GATA-3 also express AC133, another marker which defines hematopoietic cells with angioblast potential.

[0113] FIG. 2. Bone Marrow-Derived Angioblasts (BA) Have Greater Proliferative Activity In Response To Both VEGF And Ischemic Serum Than Bone Marrow-Derived Endothelial Cells (BMEC).

[0114] Depicted is the response of single-donor CD34-positive human cells sorted by a fluorescent GATA-2 mAb and cultured for 96 hours in RPMI with 20% normal rat serum, ischemic rat serum or 20 ng/ml VEGF. The numbers of CD117<sup>bright</sup>GATA-2<sup>pos</sup> and CD117<sup>dim</sup>GATA-2<sup>neg</sup> cells were quantitated by both [<sup>3</sup>H] thymidine uptake and by flow cytometry.

[0115] A. In comparison to culture in normal serum, the proliferative responses to either VEGF or ischemic serum were significantly higher for CD117<sup>bright</sup>GATA-2<sup>pos</sup> BA relative to CD117<sup>dim</sup>GATA-2<sup>neg</sup>BMEC from the same donor (both p<0.01).

[0116] B. The population expanded by culture with either VEGF or ischemic serum and characterized by multiparameter flow cytometric analysis as CD117<sup>bright</sup>GATA-2<sup>pos</sup> consisted of large blast cells, as demonstrated by high forward scatter (fsc).

[0117] C. The expanded population of CD117<sup>bright</sup>GATA-2<sup>pos</sup> cells did not demonstrate increased surface expression of mature endothelial cell markers after culture with VEGF in comparison to culture with normal medium, indicating blast proliferation without differentiation.

[0118] FIG. 3. Highly Purified Human Bone Marrow-Derived CD34 Cells Differentiate Into Endothelial Cells After in Vitro Culture.

[0119] Culture of highly-purified CD34+ human cells for 7 days in endothelial growth medium results in outgrowth of cells with morphologic and characteristic features of mature endothelial cell monolayers. The majority of the monolayers (>90%) demonstrate:

[0120] A. Exuberant cobblestone pattern of cellular proliferation and growth;

[0121] B. Uniform uptake of Dil-labeled acetylated LDL;

[0122] C. CD34 expression, as measured by immunofluorescence using a fluorescein-conjugated mAb;

[0123] D. Factor VIII expression, as measured by immunoperoxidase using a biotin-conjugated mAb; and

[0124] E. Expression of eNOS, determined by in situ hybridization using a specific probe.

[0125] FIG. 4. In vivo migratory and proliferative characteristics of bone marrow- and peripheral vasculature-derived human cells after induction of myocardial ischemia.

[0126] A-C. Intravenous injection of 2×10<sup>6</sup> Dil-labeled human CD34-enriched cells (>95% CD34 purity), CD34-negative cells (<5% CD34 purity), or saphenous vein endothelial cells (SVEC), into nude rats after coronary artery ligation and infarction. Each human cellular population caused a similar degree of infiltration in infarcted rat myocardium at 48 hours.

[0127] D. A sham procedure, with no human cells found in the non-infarcted rat heart.

- [0128] E. Measurement of human GATA-2 mRNA expression in the bone marrow and heart of infarcted rats receiving either CD34-positive cells (>95% CD34 purity), CD34-negative cells (<5% CD34 purity), normalized for total human RNA measured by GAPDH expression. GATA-2 mRNA in ischemic tissue is expressed as the fold increase above that present under the same experimental condition in the absence of ischemia. Bone marrow from ischemic rats receiving either CD34+ or CD34- cells contained similar levels of human GATA-2 mRNA, and showed a similar fold induction in GATA-2 mRNA expression after ischemia. In contrast, ischemic hearts of rats receiving CD34+ cells contained much higher levels of human GATA-2 mRNA than those receiving CD34- cells. Moreover, the degree of increase in GATA-2 mRNA expression after infarction was 2.6-fold higher for hearts infiltrated by CD34+ cells compared with CD34- cells, indicating that GATA-2+ cells within the CD34+ fraction selectively traffic to ischemic myocardium.
- [0129] F. Consecutive sections of a blood vessel within the infarct bed of a nude rat two weeks after injection of human CD34+ cells. The vessel incorporates human endothelial cells, as defined by co-expression of DiI, HLA class I as measured by immunofluorescence using a fluorescein-conjugated mAb, and factor VIII, as measured by immunoperoxidase using a biotin-conjugated mAb.
- [0130] FIG. 5. Injection of G-CSF Mobilized Human CD34+ Cells Into Rats With Acute Infarction Improves Myocardial Function.
- [0131] A-D compares the functional effects of injecting  $2 \times 10^6$  G-CSF mobilized human CD34+ (>95% purity) cells, CD34- (<5% purity) cells, peripheral saphenous vein cells, or saline, into infarcted rat myocardium.
- [0132] A. Although left ventricular ejection fraction (LVEF) was severely depressed in each group of recipients after LAD ligation, only injection of G-CSF mobilized adult human CD34+ cells was accompanied by significant, and sustained, LVEF recovery ( $p < 0.001$ ). LVEF recovery was calculated as the mean % improvement between LVEF after LAD ligation and pre-infarct LVEF.
- [0133] B. Similarly, although left ventricular end-systolic area (LVAS) was markedly increased in each group of recipients after LAD ligation, only injection of G-CSF mobilized adult human CD34+ cells was accompanied by significant, and sustained, reduction in LVAs ( $p < 0.001$ ). Reduction in LVAs was calculated as the mean % improvement between LVAs after LAD ligation and pre-infarct LVAs.
- [0134] C. Representative echocardiographic examples from each group are shown. At 48 hours after LAD ligation, diastolic function is severely compromised in each rat.
- [0135] At two weeks after injection, diastolic function is improved only in the rat receiving CD34+ cells. This effect persists at 15 weeks.
- [0136] D. At 15 weeks post-infarction, rats injected with CD34+ cells demonstrated significantly less reduction in mean cardiac index relative to normal rats than each of the other groups ( $p < 0.001$ ).
- [0137] FIG. 6. Injection Of G-CSF Mobilized Human CD34+ Cells Into Rats With Acute Infarction Induces Neo-Angiogenesis And. Modifies The Process Of Myocardial Remodeling.
- [0138] A-D depicts infarcted rat myocardium at two weeks post-LAD ligation from representative experimental and control animals stained with either hematoxylin and eosin (A,B) or immunoperoxidase following binding of anti-factor VIII mAb (C,D). E,F depicts Mason trichrome stain of infarcted rat myocardium from representative control and experimental animals at 15 weeks post-LAD ligation. G depicts between-group differences in % scar/normal left ventricular tissue at 15 weeks.
- [0139] A. Infarct zone of rat injected with human CD34+ cells demonstrates significant increase in microvasculature and cellularity of granulation tissue, numerous capillaries (arrowheads), feeding vessels (arrow), and decrease in matrix deposition and fibrosis (x200).
- [0140] B. In contrast, infarct zone of control rat injected with saline shows a myocardial scar composed of paucicellular, dense fibrous tissue (arrows) (x200).
- [0141] C. Ischemic myocardium of rat injected with human CD34+ cells demonstrates numerous factor VIII-positive interstitial angioblasts (arrows), and diffuse increase in factor VIII-positive capillaries (arrowheads) (x400).
- [0142] D. Ischemic myocardium of rat injected with saline does not contain factor VIII-positive angioblasts (arrows), and demonstrates only focal areas of granulation tissue with factor VIII positive vascularity (arrowheads) (x400).
- [0143] E. Trichrome stain of rat myocardium at 15 weeks post-infarction in rat injected with saline (x25). The collagen rich myocardial scar in the anterior wall of the left ventricle (ant.) stains blue and viable myocardium stains red. Focal islands of collagen deposition (blue) are also present in the posterior wall of the left ventricle (post). There is extensive loss of anterior wall myocardial mass, with collagen deposition and scar formation extending almost through the entire left ventricular wall thickness, causing aneurysmal dilatation and typical EKG abnormalities (persistent ST segment elevation).
- [0144] F. In contrast, trichrome stain of rat myocardium at 15 weeks post-infarction in rat receiving highly purified CD34+ cells (x25) demonstrates significantly reduced infarct zone size together with increased mass of viable myocardium within the anterior wall (ant.) and normal EKG. Numerous vessels are evident at the junction of the infarct zone and viable myocardium. There is no focal collagen deposition in the left ventricular posterior wall (post).
- [0145] G. Rats receiving CD34+ cells had a significant reduction in mean size of scar tissue relative to normal left ventricular myocardium compared with



each of the other groups ( $p < 0.01$ ). Infarct size, involving both epicardial and endocardial regions, was measured with a planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference at a given slice. For each animal, final infarct size was calculated as the average of 10-15 slices.

[0146] FIG. 7. Human Adult Bone Marrow-Derived Endothelial Precursor Cells Infiltrate Ischemic Myocardium, Inducing Infarct Bed Neoangiogenesis And Preventing Collagen Deposition.

[0147] A. Four-parameter flow cytometric phenotypic characterization of G-CSF mobilized bone marrow-derived cells removed by leukopheresis from a representative human donor adult. Only live cells were analyzed, as defined by 7-AAD staining. For each marker used, shaded areas represent background log fluorescence relative to isotype control antibody. The CD34+CD117<sup>bright</sup> subset contains a population expressing markers characteristic of primitive haemangioblasts arising during waves of murine and human embryogenesis, but not markers of mature endothelium. These cells also express CXC chemokine receptors.

[0148] B. Dil-labeled human CD34-enriched cells (>98% CD34 purity) injected intravenously into nude rats infiltrate rat myocardium after coronary artery ligation and infarction but not after sham operation at 48 hours.

[0149] C. The myocardial infarct bed at two weeks post-LAD ligation from representative rats receiving  $2.0 \times 10^6$  G-CSF mobilized human bone marrow-derived cells at 2%, 40%, or 98% CD34+ purity, and stained with either Masson's trichrome or immunoperoxidase. The infarct zones of rats receiving either 2% or 40% pure CD34+ cells show myocardial scars composed of paucicellular, dense fibrous tissue stained blue (x400). In contrast, the infarct zone of the rat injected with 98% pure human CD34+ cells demonstrates significant increase in microvasculature and cellularity of granulation tissue, numerous capillaries, and minimal matrix deposition and fibrosis (x400). Moreover, immunoperoxidase staining following binding of anti-factor VIII mAb shows that the infarct bed of the rat injected with 98% pure CD34+ cells demonstrates markedly increased numbers of factor VIII-positive capillaries, which are not seen in either of the other animals (x400).

[0150] FIG. 8. Migration Of Human Bone Marrow-Derived Endothelial Precursor Cells To The Site Of Infarction Is Dependent On Interactions Between CXCR1/2 And IL-8/Gro-Alpha Induced By Myocardial Ischemia.

[0151] A,B. Time-dependent increase in rat myocardial IL-8 and Gro-alpha mRNA expression relative to GAPDH from rats undergoing LAD ligation.

[0152] C. IL-8, Gro-alpha, and GAPDH mRNA expression at baseline, 12 hours and 48 hours after LAD ligation from a representative animal.

[0153] D. Time-dependent measurement of rat IL-8/Gro-alpha protein in serum of rats undergoing LAD

ligation. Migration of CD34+ human bone marrow-derived cells to ischemic rat myocardium is inhibited by mAbs against either rat IL-8 or the IL-8/Gro chemokine family receptors CXCR1 and CXCR2 (all  $p < 0.01$ ), but not against VEGF or its receptor Flk-1 (results are expressed as mean  $\pm$  sem of three separate experiments).

[0154] FIG. 9. CXC Chemokines Directly Induce Chemotaxis Of Bone Marrow-Derived Human CD34+ Cells To Rat Myocardium.

[0155] A and B depict results of in vitro chemotaxis of 98% pure human CD34+ cells to various conditions using a 48-well chemotaxis chamber (Neuro Probe, Md.). Chemotaxis is defined as the number of migrating cells per high power field (hpf) after examination of 10 hpf per condition tested.

[0156] A. IL-8 induces chemotaxis in a dose-dependent manner (results are expressed as mean  $\pm$  sem of three separate experiments).

[0157] B. Chemotaxis is increased in response to IL-8 and SDF-1 alpha/beta, but not VEGF or SCF.

[0158] C. Representative fluorescence microscopy demonstrating increased infiltration of intravenously-injected Dil-labeled human CD34+ cells (98% purity) into rat heart after intracardiac injection with IL-8 compared with saline injection.

[0159] D. Intracardiac injection of IL-8 at 1 mg/ml significantly increases in vivo chemotaxis of Dil-labeled human CD34+ cells (98% purity) into rat heart in comparison with injection of saline, VEGF or stem cell factor (SCF),  $p < 0.01$  (results are expressed as mean  $\pm$  sem of three separate experiments).

[0160] FIG. 10. Blocking CXCR4/SDF-1 Interactions Redirects Intravenously Injected Human CD34+ Angioblasts From Bone Marrow To Ischemic Myocardium.

[0161] A. Depicted is the response of single-donor CD34-positive human cells cultured for 96 hours in RPMI with 20% normal rat serum, ischemic rat serum or 20 ng/ml VEGF.

[0162] The numbers of CD117<sup>bright</sup>GATA-2<sup>pos</sup> cells were quantitated by both [<sup>3</sup>H] thymidine uptake and by flow cytometry. Ischemic serum induced a greater proliferative response of CD117<sup>bright</sup>GATA-2<sup>pos</sup> cells compared with each of the other conditions (both  $p < 0.01$ ).

[0163] B. The proportion of human CD34+ cells in rat bone marrow 2-14 days after intravenous injection is significantly increased after ischemia induced by LAD ligation (results are expressed as mean  $\pm$  sem of bone marrow studies in three animals at each time point).

[0164] C,D. Effects of mAbs against CXCR4, SDF-1 or anti-CD34 on trafficking of human CD34+ cells to rat bone marrow and myocardium following LAD ligation. Co-administration of anti-CXCR4 or anti-SDF-1 significantly reduced trafficking of 98% pure CD34+ cells to rat bone marrow at 48 hours and increased trafficking to ischemic myocardium (results are expressed as mean  $\pm$  sem of bone marrow

and cardiac studies performed in three LAD-ligated animals at 48 hours after injection).

**[0165] FIG. 11.** Redirected Trafficking Of Human CD34+ Angioblasts To The Site Of Infarction Prevents Remodeling And Improves Myocardial Function.

**[0166]** A,B. The effects of human CD34+ cells on reduction in LVAs (A) and improvement in LVEF (B) after myocardial infarction. Whereas injection of  $2.0 \times 10^6$  human cells containing 98% CD34+ purity significantly improved LVEF and reduced LVAs (both  $p < 0.01$ ), injection of  $2.0 \times 10^6$  human cells containing 2% and 40% CD34+ purity did not have any effect on these parameters in comparison to animals receiving saline. However, co-administration of anti-CXCR4 together with 40% pure CD34+ cells significantly improved LVEF and reduced LVAs (both  $p < 0.01$ ), to levels approaching use of cells with 98% purity.

**[0167]** C. Sections of rat hearts stained with Masson's trichrome at 15 weeks after LAD ligation and injection of  $2.0 \times 10^6$  human cells containing 2%, 40%, or 98% CD34+ purity. Hearts of rats receiving 2% and 40% pure CD34+ cells had greater loss of anterior wall mass, collagen deposition (blue), and septal hypertrophy compared with hearts of rats receiving 98% pure CD34+ cells. Co-administration of anti-CXCR4 mAb together with 40% pure CD34+ cells increased left ventricular wall mass and reduced collagen deposition.

**[0168]** D. Shows the mean proportion of scar/normal left ventricular myocardium in rats receiving >98% pure CD34+ cells or 40% pure CD34+ cells together with anti-CXCR4 mAb is significantly reduced in comparison to rats receiving 2% and 40% pure CD34+ cells ( $p < 0.01$ ) (results are expressed as mean  $\pm$  sem of three separate experiments).

**[0169] FIG. 12.** Culture of CD34+CD117<sup>bright</sup> angioblasts with serum from LAD-ligated rats increases surface expression of CCR1 and CCR2, while surface expression of CCR3 and CCR5 remains unchanged.

**[0170] FIG. 13.** Infarcted myocardium demonstrate a time-dependent increase in mRNA expression of several CCR-binding chemokines.

**[0171] FIG. 14.** Co-administration of blocking mabs against MCP-1, MCP-3, and RANTES, or against eotaxin, reduced myocardial trafficking of human angioblasts by 40-60% relative to control antibodies ( $p < 0.01$ ).

**[0172] FIG. 15.** Intracardiac injection of eotaxin into non-infarcted hearts induced 1.5-1.7 fold increase in CD34+ angioblast trafficking whereas injection of the growth factors VEGF and stem cell factor had no effect on chemotaxis despite increasing angioblast proliferation (not shown).

#### DETAILED DESCRIPTION OF THE INVENTION

**[0173]** As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

**[0174]** As used herein, "BMEC" is defined as bone marrow-derived endothelial cells.

**[0175]** As used herein, vasculogenesis is defined as the creation of new blood vessels from cells that are "pre-blood" cells such as bone marrow-derived endothelial cell precursors.

**[0176]** As used herein, mobilization is defined as inducing bone marrow-derived endothelial cell precursors to leave the bone marrow and enter the peripheral circulation. One of skill is aware that mobilized stem cells may be removed from the body by leukopheresis.

**[0177]** As used herein, ischemia is defined as inadequate blood supply (circulation) to a local area due to blockage of the blood vessels to the area.

**[0178]** As used herein, cytokine is defined as a factor that causes cells to grow or activate.

**[0179]** As used herein, chemokine is defined as a factor that causes cells to move to a different area within the body.

**[0180]** As used herein, ischemic heart disease is defined as any condition in which blood supply to the heart is decreased.

**[0181]** As used herein, "angiogenesis" is defined as the creation of blood vessels from pre-existing blood vessel cells.

**[0182]** As used herein, ischemic heart disease is defined as any condition in which blood supply to the heart is decreased.

**[0183]** As used herein, "VEGF" is defined as vascular endothelial growth factor. "VEGF-R" is defined as vascular endothelial growth factor receptor. "FGF" is defined as fibroblast growth factor. "IGF" is defined as Insulin-like growth factor. "SCF" is defined as stem cell factor. "G-CSF" is defined as granulocyte colony stimulating factor. "M-CSF" is defined as macrophage colony stimulating factor. "GM-CSF" is defined as granulocyte-macrophage colony stimulating factor. "MCP" is defined as monocyte chemoattractant protein.

**[0184]** As used herein, "CXC" chemokine refers to the structure of the chemokine. Each "C" represents a cysteine and "X" represents any amino acid.

**[0185]** As used herein, "CC" chemokine refers to the structure of the chemokine. Each "C" represents a cysteine.

**[0186]** As used herein, "recovered" means detecting and obtaining a cell based on the recoverable cell being a cell that binds a detectably labeled antibody directed against a specific marker on a cell including, but not limited to, CD117, GATA-2, GATA-3, and CD34.

**[0187]** As described herein, the chemokine administered to the subject could be in the protein form or nucleic acid form.

**[0188]** This invention provides a method of stimulating vasculogenesis in ischemia-damaged tissue of a subject comprising:

**[0189]** (a) removing stem cells from a location within the subject;

**[0190]** (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and

- [0191] (c) introducing the endothelial progenitor cells from step (b) into a different location within the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.
- [0192] In a further embodiment the endothelial progenitors are frozen for a period of time in between steps (b) and (c). In one embodiment the ischemia-damaged tissue is myocardium. In another embodiment the ischemia-damaged tissue is nervous system tissue.
- [0193] In one embodiment the endothelial progenitors are expanded by contacting the endothelial progenitors with a growth factor subsequent to step (b), but before step (c). In a further embodiment the growth factor is a cytokine. In further embodiments the cytokine is VEGF, FGF, G-CSF, IGF, M-CSF, or GM-CSF. In another embodiment the growth factor is a chemokine. In a further embodiment the chemokine is Interleukin-8. In one embodiment the endothelial progenitors are separated from other stem cells before expansion. In a further embodiment the endothelial progenitors are frozen for a period of time after expansion but before step (c).
- [0194] In one embodiment step (a) occurs prior to the subject suffering ischemia-damaged tissue and wherein step (c) occurs after the subject has suffered ischemia-damaged tissue.
- [0195] In one embodiment the stem cells are removed directly from the subject's bone marrow. In a further embodiment the stem cells are removed by aspiration from the subject's bone marrow. In one embodiment the stem cells are removed from the subject by a method comprising:
- [0196] a) introducing a growth factor into the subject to mobilize the stem cells into the subject's blood; and
- [0197] b) subsequently removing a sample of blood containing stem cells from the subject.
- [0198] In a further embodiment the growth factor is introduced subcutaneously, orally, intravenously or intramuscularly. In one embodiment the growth factor is a chemokine that induces mobilization. In a further embodiment the chemokine is Interleukin-8. In one embodiment the growth factor is a cytokine. In a further embodiment the cytokine is G-CSF, M-CSF, or GM-CSF.
- [0199] This invention also provides the instant method, wherein the endothelial progenitor cells are recovered based upon their expression of CD117.
- [0200] This invention also provides the instant method, wherein the endothelial progenitor cells are recovered based upon their expression of a GATA-2 activated gene product. In one embodiment the gene product is selected from the following group: preendothelin-1, big endothelin, endothelin-1.
- [0201] In one embodiment the endothelial progenitors express GATA-2, and the endothelial progenitors are recovered as such by detection of intracellular GATA-2 expression or GATA-2 activity in those cells.
- [0202] In one embodiment the subject has suffered or is suffering from one or more of the following: myocardial infarction, chronic heart failure, ischemic heart disease, coronary artery disease, diabetic heart disease, hemorrhagic stroke, thrombotic stroke, embolic stroke, limb ischemia or another, disease in which tissue is rendered ischemic.
- [0203] In one embodiment the endothelial progenitors are introduced into the subject by injection directly into the peripheral circulation, heart muscle, left ventricle, right ventricle, coronary artery, cerebro-spinal fluid, neural tissue, ischemic tissue or post-ischemic tissue.
- [0204] In one embodiment the method further comprises administering to the subject one or more of the following: an inhibitor of Plasminogen Activator Inhibitor, Angiotensin Converting Enzyme Inhibitor or a beta blocker, wherein such administration occurs prior to, concomitant with, or following step (c).
- [0205] This invention also provides a method of stimulating angiogenesis in peri-infarct tissue in a subject comprising:
- [0206] (a) removing stem cells from a location within a subject;
- [0207] (b) recovering endothelial progenitor cells from the stem cells removed in step (a);
- [0208] (c) expanding the endothelial progenitor cells recovered in step (b) by contacting the progenitor cells with a growth factor; and
- [0209] (d) introducing the expanded endothelial progenitor cells from step (c) into a different location in the subject such that the endothelial progenitor cells stimulate angiogenesis in peri-infarct tissue in the subject.
- [0210] This invention also provides a method of selectively increasing the trafficking of endothelial progenitor cells to ischemia-damaged tissue in a subject comprising:
- [0211] (a) administering endothelial progenitor cells to a subject; and
- [0212] (b) administering a chemokine to the subject so as to thereby attract the endothelial progenitor cells to the ischemia-damaged tissue.
- [0213] In one embodiment the chemokine is administered to the subject prior to administering the endothelial progenitors. In an alternative embodiment the chemokine is administered to the subject concurrently with the endothelial progenitors. In an alternative embodiment the chemokine is administered to the subject after administering the endothelial progenitors. In one embodiment the chemokine is a CXC chemokine. In a further embodiment the CXC chemokine is selected from the group consisting of Interleukin-8, Gro-Alpha, or Stromal-Derived Factor-1. In one embodiment the chemokine is a CC chemokine. In a further embodiment the CC chemokine is selected from the group consisting of RANTES, EOTAXIN, MCP-1, MCP-2, MCP-3, or MCP-4.
- [0214] In one embodiment the chemokine is administered to the subject by injection into peripheral circulation, heart muscle, left ventricle, right ventricle, coronary arteries, cerebro-spinal fluid, neural tissue, ischemic tissue or post-ischemic tissue.
- [0215] This invention also provides a method of increasing trafficking of endothelial progenitor cells to ischemia-

damaged tissue in a subject comprising inhibiting any interaction between Stromal-Derived Factor-1 and CXCR4.

[0216] In one embodiment the interaction between Stromal-Derived Factor-1 (SDF-1) and CXCR4 is inhibited by administration of an anti-SDF-1 or an anti-CXCR4 monoclonal antibody to the subject. In one embodiment the instant method further comprises administering to the subject ACE inhibitor, AT<sub>1</sub>-receptor blocker, or beta blocker. ng enzyme inhibitor, an AT<sub>1</sub>-receptor blocker, or a beta blocker.

[0217] This invention also provides a method of reducing trafficking of endothelial progenitor cells to bone marrow in a subject comprising inhibiting production of Stromal-Derived Factor-1 in the subject's bone marrow. In one embodiment the SDF-1 production is inhibited by administration of an anti-SDF-1 or anti-CXCR4 monoclonal antibody to the subject.

[0218] This invention also provides a method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific chemokine produced by proliferating cells associated with the cancer so as to reduce trafficking of endothelial progenitor cells to such proliferating cells and thereby treat the cancer in the subject.

[0219] This invention also provides a method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific receptor located on an endothelial progenitor cell, for a chemokine produced by proliferating cells associated with the cancer, so as to reduce trafficking of the endothelial progenitor cell to such proliferating cells and thereby treat the cancer in the subject.

[0220] This invention also provides a method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce vasculogenesis in the subject's tumor and thereby treat the tumor.

[0221] This invention also provides a method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce angiogenesis in the subject's tumor and thereby treat the tumor.

[0222] This invention also provides a method for expressing a gene of interest in an endothelial progenitor cell or a mast progenitor cell which comprises inserting into the cell a vector comprising a promoter containing a GATA-2 motif and the gene of interest.

[0223] This invention also provides the instant method, wherein the vector is inserted into the cell by transfection.

[0224] This invention also provides the instant method, wherein the promoter is a preproendothelin-1 promoter.

[0225] This invention also provides the instant method, wherein the promoter is of mammalian origin.

[0226] This invention also provides the instant method, wherein the promoter is of human origin.

[0227] This invention provides a composition comprising an amount of a monoclonal antibody directed against an

epitope of a specific chemokine produced by a cancer effective to reduce trafficking of endothelial progenitor cells to the cancer, and a pharmaceutically acceptable carrier.

[0228] This invention provides a method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

[0229] (a) removing stem cells from a location within the subject;

[0230] (b) recovering endothelial progenitor cells from the stem cells removed in step (a);

[0231] (c) recovering those endothelial progenitor cells recovered in step (b) that express GATA-2;

[0232] (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and

[0233] (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality.

[0234] In one embodiment the abnormality is ischemia-damaged tissue. In one embodiment the gene product is proendothelin. In one embodiment the gene product is endothelin. In one embodiment the subject is a mammal. In a further embodiment the mammal is a human

[0235] This invention provides a method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

[0236] (a) removing stem cells from a location within the subject;

[0237] (b) recovering mast progenitor cells from the stem cells removed in step (a);

[0238] (c) recovering those mast progenitor cells recovered in step (b) that express GATA-2;

[0239] (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and

[0240] (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality

[0241] In one embodiment the abnormality is ischemia-damaged tissue. In one embodiment the gene product is proendothelin. In one embodiment the gene product is endothelin. In one embodiment the subject is a mammal. In a further embodiment the mammal is a human

[0242] This invention provides the a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising:

[0243] (a) removing stem cells from a location in the subject;

[0244] (b) recovering cells that express CD117 from the stem cells; and

- [0245] (c) introducing the recovered cells into a different location in the subject such that the cells improve myocardial function in the subject.
- [0246] In one embodiment the subject is a mammal. In a further embodiment the mammal is a human.
- [0247] This invention also provides a method of stimulating vasculogenesis in ischemia-damaged tissue in a subject comprising:
- [0248] (a) obtaining allogeneic stem cells;
  - [0249] (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and
  - [0250] (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.
- [0251] In alternative embodiments the allogeneic stem cells are removed from embryonic, fetal or cord blood sources.
- [0252] This invention provides a method of stimulating angiogenesis in ischemia-damaged tissue in a subject comprising:
- [0253] (a) obtaining allogeneic stem cells;
  - [0254] (b) recovering endothelial progenitor cells in the stem cells removed in step (a); and
  - [0255] (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate angiogenesis in the subject's ischemia-damaged tissue.
- [0256] In alternative embodiments the allogeneic stem cells are removed from embryonic, fetal or cord blood sources.
- [0257] This invention also provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting G-CSF into the subject in order to mobilize endothelial progenitor cells.
- [0258] This invention also provides a method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting anti-CXCR4 antibody into the subject. In one embodiment the method further comprises introducing endothelial progenitors into the subject. In one embodiment the method further comprises introducing G-CSF into the subject in order to mobilize endothelial progenitors.
- [0259] This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

## EXPERIMENTAL DETAILS

### First Series of Experiments

#### Experimental Procedures and Results

- [0260] 1. Mobilization and Identification of Bone Marrow-derived Cells
- [0261] Following G-CSF mobilization, 60-80% of highly purified human CD34 cells (>90% positive) co-expressed

the stem cell factor receptor CD117, FIG. 1a, of which 15-25% expressed CD117 brightly and 75-85% expressed CD117 dimly. By quadruple parameter analysis, two populations of CD34 cells were recovered which expressed VEGFR-2 (Flk-1), one accounting for 20-30% of CD117<sup>dim</sup> cells and expressing high levels of VEGFR-2, and a second accounting for 10-15% of CD117<sup>bright</sup> cells and expressing lower levels of VEGFR-2, FIG. 1b. The VEGFR-2 positive cells within the CD34+CD117<sup>dim</sup>

[0262] population, but not those within the CD34+CD117<sup>bright</sup> subset, displayed phenotypic characteristics of mature, vascular endothelium, including high level expression of Tie-2, eNOS, vWF, E-selectin (CD62E), and ICAM (CD54). In contrast, as shown in FIG. 1c, the VEGFR-2 positive cells within the CD34+CD117<sup>dim</sup> subset, but not those within the CD34+CD117<sup>bright</sup> subset, expressed markers characteristic of primitive hemangioblasts arising during waves of murine and human embryogenesis, including GATA-2, GATA-3, and low levels of Tie-2. Moreover, CD117<sup>bright</sup> cells which co-expressed GATA-2 and GATA-3 were also strongly AC133 positive, another marker which has recently been suggested to define a hematopoietic population with angioblast potential (2), figure id. However, since AC133 expression was also detected on a subset of CD117<sup>dim</sup> cells which was negative for GATA-2 and GATA-3, we conclude that identification of an embryonic bone-marrow derived angioblast (BA) phenotype requires concomitant expression of GATA-2, GATA-3, and CD117<sup>bright</sup> in addition to AC133. Thus, G-CSF treatment mobilizes into the peripheral circulation a prominent population of mature, bone marrow-derived endothelial cells (BMEC), and a smaller bone marrow-derived population with phenotypic characteristics of embryonic angioblasts (BA).

#### [0263] 2. Expansion of Bone Marrow-derived Cells

[0264] Since the frequency of circulating endothelial cell precursors in animal models has been shown to be increased by either VEGF (27) or regional ischemia (10-13), we next compared the proliferative responses of BA and BMEC to VEGF and to factors in ischemic serum (28). As shown in FIG. 2a, following culture for 96 hours with either VEGF or ischemic serum, CD117<sup>bright</sup>GATA-2<sup>pos</sup> BA demonstrated significantly higher proliferative responses relative to CD117<sup>pos</sup>GATA-2<sup>neg</sup> BMEC from the same donor. For VEGF, BA showed 2.9-fold increase in proliferation above baseline compared with 1.2-fold increase for BMEC,  $p < 0.01$ , while for ischemic serum from Lew rats with myocardial infarction BA showed 4.3-fold increase in proliferation above normal serum compared with 1.7-fold increase for BMEC,  $p < 0.01$ . Culture with either VEGF or ischemic serum greatly expanded the BA population of large blast cells, FIG. 2b, which continued to express immature markers, including GATA-2, GATA-3, and CD117<sup>bright</sup>, but not markers of mature endothelial cells, FIG. 2c, indicating blast proliferation without differentiation. Following culture of CD34-positive monolayers on fibronectin in endothelial growth medium for 7 days (29), an exuberant cobblestone pattern of proliferation was seen, FIG. 3a, with the majority of the adherent monolayers (>95%) having features characteristic of endothelial cells, FIG. 3b-e, including uniform uptake of acetylated LDL, and co-expression of CD34, factor VIII, and eNOS. Since the BMEC population had low

proliferative responses to VEGF or cytokines in ischemic serum, the origin of the exuberant endothelial cell outgrowth in culture is most likely the BA population defined by surface expression for GATA-2, GATA-3, and CD117<sup>bright</sup>.

**[0265] 3. In vivo Migration of Bone Marrow-derived CD34+ Cells to Sites of Regional Ischemia**

**[0266]** Next we compared the in vivo migratory and proliferative characteristics of bone marrow- and peripheral vasculature-derived human cells after induction of regional ischemia. As shown in FIG. 4a-c, intravenous injection of  $2 \times 10^6$  Dil-labeled human CD34-positive cells (>95% CD34+ purity), CD34-negative cells (<5% CD34+ purity), or saphenous vein endothelial cells (SVEC), into nude rats after coronary artery ligation and infarction resulted in similar degree of infiltration in rat myocardium at 48 hours (30). The trafficking was specifically directed to the infarct area since few Dil-labeled cells were detected in unaffected areas of hearts with regional infarcts, not shown, and neither G-CSF mobilized CD34+ cells nor mature human endothelial cells infiltrated normal myocardium, FIG. 4d. Although similar numbers of CD34+ and CD34- cells-migrated to ischemic myocardium, the proportional increase in human GATA-2 mRNA expression in ischemic myocardium relative to normal myocardium (31) was 2.6-fold greater following injection of highly CD34-enriched cells compared with CD3430 cells ( $p < 0.001$ ), FIG. 4e. Moreover, blood vessels which incorporated human endothelial cells, as defined by co-expression of Dil, HLA class I, and factor VII, could be detected two weeks after injection of human CD34+ cells, but not after injection of CD34- cells or SVEC, FIG. 4f. Together, these results indicate that adult bone marrow-derived human CD34+ cells contain a population which selectively responds to in vivo signals from sites of regional ischemia with augmented migration, localization, and endothelial differentiation.

**[0267] 4. Effects of Injection of G-CSF mobilized Human CD34+ Cells Into Infarcted Rat Myocardium**

**[0268]** We next compared the functional effects of injecting G-CSF mobilized human CD34+ (>95%) cells, CD34- (<5%) cells, peripheral saphenous vein cells, or saline, into infarcted rat myocardium. After ILAD ligation, left ventricular function was severely depressed in each group of recipients, with left ventricular ejection fraction (LVEF) being reduced by means of 25-43% and left ventricular end-systolic area being increased by means of 44-90%, FIG. 5a and b. Remarkably, within two weeks of injecting G-CSF mobilized adult human CD34+ cells, LVEF recovered by a mean of  $22 \pm 6\%$  ( $p < 0.001$ ), FIG. 5a. This effect was long-lived, and increased by the end of follow-up, 15 weeks, to  $34 \pm 4\%$ . In contrast, injection of G-CSF mobilized human CD34- cells, saphenous vein endothelial cells, or saline, had no effect on LVEF. In a parallel fashion, injection of G-CSF mobilized human CD34+ cells reduced left ventricular end-systolic area by a mean of  $26 \pm 8\%$  by 2 weeks and  $37 \pm 6\%$  by 15 weeks, whereas none of the other recipient groups demonstrated such effect ( $p < 0.001$ ), FIG. 5b. Representative echocardiographic examples for each group are shown in FIG. 5c. Moreover, at 15 weeks post-infarction mean cardiac index in rats injected with CD34+ cells was only reduced by  $26 \pm 8\%$  relative to normal rats, whereas mean cardiac index for each of the other groups was reduced by 48-59% ( $p < 0.001$ ), FIG. 5d.

**[0269]** Histologic examination at two weeks post-infarction (33) revealed that injection of CD34+ cells was accompanied by significant increase in microvasculature and cellularity of granulation tissue, and decrease in matrix deposition and fibrosis within the infarct zone in comparison to controls, FIG. 6a and b. Moreover, ischemic myocardium of rats injected with human CD34+ cells contained significantly greater numbers of factor VIII-positive interstitial angioblasts and capillaries in comparison to ischemic myocardium of control rats, FIG. 6c and d. Quantitation of capillary numbers demonstrated a significant increase in neo-angiogenesis within the infarct zone of rats who received CD34+ cells (mean number of factor VIII-positive capillaries per high power field  $92 \pm 5$  vs  $51 \pm 4$  in saline controls,  $p < 0.01$ ), but not within normal myocardium ( $36 \pm 2$  vs  $37 \pm 3$  capillaries per high power field). No increase in capillary numbers were observed in ischemic rat myocardium infiltrated with CD34- cells or SVEC. At 15 weeks post-infarction, rats receiving highly purified CD34+ cells demonstrated significantly reduced infarct zone sizes together with increased mass of viable myocardium within the anterior free wall compared to each of the other groups, FIG. 6e and f. Numerous vessels were evident at the junction of the infarct zone and viable myocardium in tissues infiltrated with CD34+ cells. Whereas collagen deposition and scar formation extended almost through the entire left ventricular wall thickness in controls, with aneurysmal dilatation and typical EKG abnormalities, the infarct scar extended only to 20-50% of the left ventricular wall thickness in rats receiving CD34+ cells. Moreover, pathological collagen deposition in the non-infarct zone was markedly reduced in rats receiving CD34+ cells. Overall, the mean proportion of scar/normal left ventricular myocardium was 13% in rats receiving CD34+ cells compared with 36-45% for each of the other groups ( $p < 0.01$ ), FIG. 6g.

### Discussion

**[0270]** The experiments described above demonstrate that neo-angiogenesis of the infarct bed by human bone marrow-derived endothelial cell precursors prevents scar development, maintains viable myocardium, and improves ventricular function in a rodent model of myocardial ischemia. Following infarction, the viable myocardial tissue bordering the infarct zone undergoes a significant degree of hypertrophy (5,34-35). Although neoangiogenesis within the infarcted tissue appears to be an integral component of the remodeling process (36,37), under normal circumstances the capillary network cannot keep pace with tissue growth and is unable to support the greater demands of the hypertrophied, but viable, myocardium which subsequently undergoes apoptosis due to inadequate oxygenation and nutrient supply. The development of neoangiogenesis within the myocardial infarct scar appears to require activation of latent collagenase and other proteinases following plasminogen activation by urokinase-type plasminogen activator (u-PA) expressed on infiltrating leukocytes (38). The importance of bone marrow-derived endothelial precursors in this process has been demonstrated in u-PA-/- mice where transplantation of bone marrow from congenic wild-type strains restored defective myocardial revascularization post-infarction (38). Since u-PA mRNA transcription and proteolytic activity in human mononuclear cells and tumor cell lines is significantly increased by the colony stimulating factors G-CSF, M-CSF, and GM-CSF (39-41), this provides a rationale for

in vivo or ex vivo use of these cytokines to mobilize and differentiate large numbers of human adult bone marrow-derived angioblasts for therapeutic revascularization of the infarct zone.

[0271] Cell surface and RNA expression of the transcription factor GATA-2 appears to selectively identify human adult bone marrow-derived angioblasts capable of responding to signals from ischemic sites by proliferating and migrating to the infarct zone, and subsequently participating in the process of neo-angiogenesis. Of particular interest, GATA-2 is a co-factor for endothelial cell transcription of preproendothelin-1 (ppET-1) (42), the precursor molecule of the potent vasoconstrictor and hypertrophic autocrine peptide ET-1. Since ppET-1 transcription is also increased by angiotensin II (43), produced as a result of activation of the renin-angiotensin neurohormonal axis following myocardial infarction, the angioblasts infiltrating the infarct bed may be secreting high levels of ET-1 due to the synergistic actions of angiotensin II surface receptor signalling and GATA-2 transactivation. The observation that newly-formed vessels within the infarct scar have thicker walls, lower vasodilator responses to stronger vasoactive substances than vessels within normal myocardium (44) are consistent with effects of increased autocrine ET-1 activity, and support the possibility that neo-angiogenic vasculature is derived from infiltrating GATA-2 positive angioblasts.

[0272] Together, the results of the above-described experiments indicate that injection of G-CSF mobilized adult human CD34+ cells with phenotypic and functional properties of embryonic hemangioblasts can stimulate neo-angiogenesis in the infarct vascular bed, thus reducing collagen deposition and scar formation in myocardial infarction. Although the degree of reduction in myocardial remodeling as a result of neoangiogenesis was striking, further augmentation in myocardial function might be achieved by combining infusion of human angioblasts with ACE inhibition or AT<sub>1</sub>-receptor blockade to reduce angiotensin II-dependent cardiac fibroblast proliferation, collagen secretion, and plasminogen activator-inhibitor (PAI) production (45, 46). The use of cytokine-mobilized autologous human bone-marrow angioblasts for revascularization of myocardial infarct tissue, in conjunction with currently used therapies (47-49), offers the potential to significantly reduce morbidity and mortality associated with left ventricular remodeling post-myocardial infarction.

## Second Series of Experiments

### Methods

[0273] 1. Purification of Cytokine-mobilized Human CD34+ Cells

[0274] Single-donor leukopheresis products were removed from humans treated with recombinant G-CSF 10 mg/kg (Amgen, Calif.) sc daily for four days. Mononuclear cells were separated by Ficoll-Hypaque, and highly-purified CD34+ cells (>98% positive) were removed using magnetic beads coated with anti-CD34 monoclonal antibody (mAb) (Miltenyi Biotec Ltd, Calif.). Purified CD34 cells were stained with fluorescein-conjugated mAbs against CD34, CD117, VEGFR-2, Tie-2, GATA-2, GATA-3, AC133, vWF, eNOS, CD54, CD62E, CXCR1, CXCR2, CXCR4, and analyzed by four-parameter fluorescence using FACScan (Becton Dickinson, Calif.).

[0275] 2. Proliferative Studies of Human Endothelial Progenitors

[0276] Single-donor CD34-positive cells were cultured for 96 hours in RPMI with either 20% normal rat serum, ischemic rat serum or 20 ng/ml VEGF, then pulsed with [<sup>3</sup>H] thymidine (Amersham Life Science Inc, IL, USA) (1 mCi/well) and uptake was measured in an LK Betaplate liquid scintillation counter (Wallace, Inc., Gaithersburg, Md.). The proportion of CD117<sup>bright</sup>GATA-2<sup>pos</sup> cells after 96 hours of culture in each condition was also quantitated by flow cytometry.

[0277] 3. Chemotaxis of Human Bone Marrow-derived Endothelial Progenitors

[0278] Highly-purified CD34+ cells (>98% positive) were plated in 48-well chemotaxis chambers fitted with membranes (8 mm pores) (Neuro Probe, Md.). After incubation for 2 hours at 37°, chambers were inverted and cells were cultured for 3 hours in medium containing IL-8 at 0.2, 1.0 and 5.0 mg/ml, SDF-1 alpha/beta 1.0 mg/ml, VEGF and SCF. The membranes were fixed with methanol and stained with Leukostat (Fischer Scientific, Illinois). Chemotaxis was calculated by counting migrating cells in 10 high-power fields.

[0279] 4. Animals, Surgical procedures, Injection of Human Cells, and Quantitation of Cellular Migration Into Tissues

[0280] Rowett (rnu/rnu) athymic nude rats (Harlan Sprague Dawley, Indianapolis, Ind.) were used in studies approved by the "Columbia University Institute for Animal Care and Use Committee". After anesthesia, a left thoracotomy was performed, the pericardium was opened, and the left anterior descending (LAD) coronary artery was ligated. Sham-operated rats had a similar surgical procedure without having a suture placed around the coronary artery. 48 hours after LAD ligation 2.0×10<sup>6</sup> Dil-labeled human CD34+ cells (>95%, 40%, <2% purity) removed from a single donor after G-CSF mobilization were injected into the tail vein in the presence or absence of mAbs with known inhibitory activity against CXCR1, CXCR2, CXCR4, CD34, rat IL-8 (Immunolaboratories, Japan) and rat SDF-1α & D Systems, Minnesota), or isotype control antibodies. Control animals received saline after LAD ligation. Each group consisted of 6-10 rats. Quantitation of myocardial infiltration after injection of human cells was performed by assessment of Dil fluorescence in hearts from rats sacrificed 2 days after injection (expressed as number of Dil-positive cells per high power field, minimum 5 fields examined per sample). Quantitation of rat bone marrow infiltration by human cells was performed in 12 rats at baseline, days 2, 7, and 14 by flow cytometric and RT-PCR analysis of the proportion of HLA class I-positive cells relative to the total rat bone marrow population.

[0281] 5. Analyses of Myocardial Function

[0282] Echocardiographic studies were performed at baseline, 48 hours after LAD ligation, and at 2, 6 and 15 weeks after injection of cells or saline, using a high frequency liner array transducer (SONOS 5500, Hewlett Packard, Andover, Mass.). 2D images were removed at mid-papillary and apical levels. End-diastolic (EDV) and end-systolic (ESV) left ventricular volumes were removed by bi-plane area-length method, and % left ventricular ejection fraction

(LVEF) was calculated as  $[(EDV-ESV)/EDV] \times 100$ . Left ventricular area at the end of systole (LVAs) was measured by echocardiography at the level of the mitral valve. LVEF recovery and reduction in LVAs were calculated as the mean improvement between the respective values for each at different time points after LAD ligation relative to pre-infarct values.

#### [0283] 6. Histology and Immunohistochemistry

[0284] Histologic studies were performed on explanted rat hearts at 2 and 15 weeks after injection of human cells or saline. Following excision, left ventricles from each experimental animal were sliced at 10-15 transverse sections from apex to base. Representative sections were put into formalin for histological examination, stained freshly with anti-factor VIII mAb using immunoperoxidase technique to quantitate capillary density, or stained with Masson trichrome and mounted. The lengths of the infarcted surfaces, involving both epicardial and endocardial regions, were measured with a planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference. Final infarct size was calculated as the average of all slices from each heart.

#### [0285] 7. Measurement of Rat CXC Chemokine mRNA and Protein Expression

[0286] Poly(A)<sup>+</sup> mRNA was extracted by standard methods from the hearts of 3 normal and 12 LAD-ligated rats. RT-PCR was used to quantify myocardial expression of rat IL-8 and Gro- $\alpha$  mRNA at baseline and at 6, 12, 24 and 48 hours after LAD ligation after normalizing for total rat RNA as measured by GAPDH expression. After priming with oligo (dT) 15-mer and random hexamers, and reverse transcribed with Monoley murine lymphotropic virus reverse transcriptase (Invitroaen, Carlsbad, Calif., USA), cDNA was amplified in the polymerase chain reaction (PCR) using Taq polymerase (Invitrogen, Carlsbad, Calif., USA), radiolabeled dideoxy-nucleotide ( $[\alpha^{32}P]$ -ddATP: 3,000 Ci/mmol, Amersham, Arlington Heights, Ill.), and primers for rat IL-8, Gro- $\alpha$  and GAPDH (Fisher Genosys, Calif.). Primer pairs (sense/antisense) for rat IL-8, Gro- $\alpha$  AND GAPDH were, gaagatagattgcacccgatg (SEQ ID NO:1)/catagcctctcacatttc (SEQ ID NO:2), ggcgcgcctccgcaatgagctgcgc (SEQ ID NO:3)/cttggggacacccctcagcatctttgg (SEQ ID NO:4), and ctctaccacggcaagttaa (SEQ ID NO:5)/gggatgacctgcccacagc (SEQ ID NO:6), respectively. The labeled samples were loaded into 2% agarose gels, separated by electrophoresis, and exposed for radiography for 6 h at -70°. Serum levels of rat IL-8/Gro- $\alpha$  were measured at baseline and at 6, 12, 24 and 48 hours after LAD ligation in four rats by a commercial ELISA using polyclonal antibodies against the rat IL-8/Gro homologue CINC (Immuno-Laboratories, Japan). The amount of protein in each serum sample was calculated according to a standard curve of optical density (OD) values constructed for known levels of rat IL-8/Gro- $\alpha$  protein.

#### Experimental Procedures and Results

##### [0287] 1. Selective Trafficking of Endothelial Precursors

[0288] Following immunoselection of G-CSF mobilized human CD34 cells to >98% purity, 60-80% co-expressed the stem cell factor receptor CD117. By quadruple parameter analysis, FIG. 7a, 10-15% of CD117<sup>bright</sup> cells were found

to express a phenotype characteristic of embryonic angioblasts, with low level surface expression of VEGFR-2 and Tie-2, as well as the transcription factors GATA-2 and GATA-3, and AC133, recently shown to identify endothelial precursors (79). These cells did not express markers of mature endothelial cells such as vWF, eNOS and E-selectin, but were positive for the CXC chemokine receptors 1, 2, and 4. Intravenous injection of  $2 \times 10^6$  DiI-labeled human CD34<sup>+</sup> cells (>98%, 40%, and 2% purity) into LAD-ligated Rowett nude rats was accompanied at 48 hours by dense infiltration of rat myocardium, FIG. 7b. The trafficking of these cells was specifically directed to the infarct area since few DiI-labeled cells were detected in unaffected areas of hearts with regional infarcts, not shown, and DiI-labeled cells did not infiltrate myocardium from sham-operated rats, FIG. 7b. By two weeks post-injection, rats receiving >98% pure human CD34<sup>+</sup> cells demonstrated increased infarct bed microvasculature and reduced matrix deposition and fibrosis, FIG. 7c. The number of factor VIII-positive capillaries per high power field was over three-fold higher in the infarct bed of rats receiving  $2 \times 10^6$  cells containing >98% pure CD34<sup>+</sup> purity than in the analogous region in rats receiving  $2 \times 10^6$  cells containing either 2% or 40% CD34<sup>+</sup> purity,  $p < 0.01$ , FIG. 7c. Moreover, the majority of these capillaries were of human origin since they expressed HLA class I molecules (not shown). Thus, although various populations of human bone marrow-derived cells migrate to the infarct bed, vasculogenesis appears to require selective trafficking of a critical number of endothelial precursors.

##### [0289] 2. Effects of Ischemia on CXC Chemokine Production by Infarcted Myocardium

[0290] Since human leukocyte chemotaxis and tissue infiltration is regulated by interactions between specific chemokines and CXC cell surface receptors, we next investigated the effects of ischemia on CXC chemokine production by infarcted rat myocardium. As shown in FIG. 8a-c, infarcted myocardium demonstrated a time-dependent increase in mRNA expression of the CXCR1/2-binding ELR-positive chemokines IL-8 and Gro- $\alpha$ , with maximal expression at 6-12 hours after LAD ligation. In comparison to non-infarcted myocardium, tissues after LAD ligation expressed 7.2-7.5 fold higher mRNA levels of these ELR-positive pro-angiogenic chemokines after normalizing for total mRNA content ( $p < 0.001$ ). Moreover, serum IL-8 levels increased by 8-10 fold within 6-12 hours after LAD ligation ( $p < 0.001$ ), and remained elevated at 48 hours, FIG. 8d. Co-administration of blocking mAbs against either IL-8 and Gro- $\alpha$ , or against the surface receptors for these pro-angiogenic chemokines, CXCR1 or CXCR2, reduced myocardial trafficking of human angioblasts by 40-60% relative to control antibodies ( $p < 0.01$ ), FIG. 8e.

##### [0291] 3. Chemotactic Responses of Human Bone Marrow-derived CD34<sup>+</sup> Angioblasts to Chemokines.

[0292] In subsequent experiments we directly measured in vitro and in vivo chemotactic responses of human bone marrow-derived CD34<sup>+</sup> angioblasts to IL-8. As shown in FIG. 9a, in vitro chemotaxis of human CD34<sup>+</sup> cells was induced by IL-8 in a dose-dependent manner, with concentrations between 0.2-5  $\mu$ M. The ELR- chemokine SDF-1, produced constitutively by bone marrow stromal cells, induced a similar degree of chemotaxis of CD34<sup>+</sup> cells at concentrations similar to IL-8, FIG. 9b. In contrast, chemo-



taxis was not induced by the growth factors VEGF or stem cell factor (SCF). Moreover, intracardiac injection of IL-8 at 1  $\mu\text{g}/\text{ml}$  into non-infarcted hearts induced *in vivo* chemotaxis of CD34+ cells, FIG. 9c, whereas neither VEGF nor SCF, used as controls, had any chemotactic effect *in vivo*, FIG. 9d. Together, these results indicate that increased tissue expression of ELR-positive chemokines augments vasculogenesis *in vivo* by inducing chemotaxis of bone marrow-derived endothelial precursor cells to sites of tissue ischemia.

**[0293] 4. Interruption of CXCR4/SDF-1 Interactions to Redirect Trafficking of Human CD34-Positive Cells from Bone Marrow to Myocardium.**

**[0294]** In addition to augmenting trafficking of intravenously injected human CD34+ angioblasts to damaged myocardium, ischemic serum from LAD-ligated rats caused rapid expansion of the circulating CD34+CD117<sup>bright</sup> angioblast population and concomitantly increased trafficking of these cells to the bone marrow. As shown in FIG. 10a, culture for 2 days with either VEGF or ischemic serum increased proliferation of CD34+CD117<sup>bright</sup> angioblasts by 2.8 and 4.3 fold, respectively ( $p < 0.01$ ). Moreover, as shown in FIG. 10b, bone marrow from ischemic rats after LAD ligation contained 5-8 fold higher levels of human CD34+CD117<sup>bright</sup> angioblasts compared with bone marrow from normal rats 2-14 days after intravenous injection of  $2 \times 10^6$  human CD34-positive cells (>95% purity), ( $p < 0.001$ ). Since SDF-1 is constitutively expressed by bone marrow stromal cells and preferentially promotes bone marrow migration of circulating CD34+ cells which are actively cycling (80), we investigated whether the increased homing of human CD34+CD117<sup>bright</sup> angioblasts to ischemic rat bone marrow was due to heightened SDF-1/CXCR4 interactions. As shown in FIG. 10c, co-administration of mAbs against either human CXCR4 or rat SDF-1 significantly inhibited migration of intravenously administered CD34+ human angioblasts to ischemic rat bone marrow by compared with anti-CD34 control antibody (both  $p < 0.001$ ). Moreover, co-administration of mAbs against either human CXCR4 or rat SDF-1 increased trafficking of CD34+ human angioblasts to ischemic rat myocardium by a mean of 24% and 17%, respectively (both  $p < 0.001$ ), FIG. 10d. By two weeks, the myocardial infarct bed of rats receiving human CD34+ cells in conjunction with anti-CXCR4 mAb demonstrated >3-fold increase in microvasculature compared with those receiving CD34+ cells in conjunction with isotype control antibody. These results indicate that although intravenously injected CD34+ angioblasts traffic to infarcted myocardium and induce vasculogenesis in response to augmented production of ELR+ chemokines, the efficiency of this process is significantly reduced by concomitant angioblast migration to the bone marrow in response to SDF-1. Interruption of CXCR4/SDF-1 interactions redirects trafficking of the expanded, cycling population of human CD34-positive cells from bone marrow to myocardium after infarction, increasing infarct bed neoangiogenesis.

**[0295] 5. Improvement in Myocardial Function**

**[0296]** Although left ventricular function was severely depressed after LAD ligation, injection of >98% pure CD34+ cells was associated with significant recovery in left ventricular size and function within two weeks, and these effects persisted for the entire 15 week period of follow-up,

FIG. 11a and b. In rats receiving >98% pure CD34+ cells, left ventricular end-systolic area decreased by a mean of  $37 \pm 6\%$  by 15 weeks compared to immediately post-infarction, FIG. 11a, and left ventricular ejection fraction (LVEF) recovered by a mean of  $34 \pm 4\%$  by 15 weeks ( $p < 0.001$ ), FIG. 11b ( $p < 0.001$ ). Improvement in these parameters depended on the number of CD34+ cells injected, since intravenous injection of  $2 \times 10^6$  G-CSF mobilized human cells containing 2% or 40% CD34+ purity did not significantly improve myocardial function despite similar degrees of trafficking to ischemic myocardium, FIGS. 11a and b. However, co-administration of anti-CXCR4 mAb together with G-CSF mobilized human bone marrow-derived cells containing 40% CD34+ purity significantly improved LVEF recovery and reduced LVAs, to levels seen with >98% CD34+ purity. By trichrome stain, significant differences in left ventricular mass and collagen deposition were observed between the groups, FIG. 11c. In rats receiving  $2 \times 10^6$  human cells containing 2% CD34+ purity, the left ventricular anterior wall was completely replaced by fibrous tissue and marked compensatory septal hypertrophy was present. Similar changes were seen in hearts of rats receiving  $2 \times 10^6$  human cells containing 40% CD34+ purity. In contrast, in hearts of rats receiving  $2 \times 10^6$  human cells containing 98% CD34+ purity significantly greater anterior wall mass was maintained, with normal septal size and minimal collagen deposition. Of particular interest, hearts of rats receiving  $2 \times 10^6$  human cells containing 40% purity together with anti-CXCR4 mAb demonstrated similar increase in anterior myocardial wall mass, decrease in septal hypertrophy, and reduction in collagen deposition. Overall, the mean proportion of fibrous scar/normal left ventricular myocardium was 13% and 21%, respectively, in rats receiving >98% pure CD34+ cells or 40% pure CD34+ cells together with anti-CXCR4 mAb, compared with 36-45% for rats receiving 2% and 40% pure CD34+ cells ( $p < 0.01$ ), FIG. 11d. Thus, augmentation of infarct bed vasculogenesis by increasing selective trafficking of a critical number of endothelial precursors leads to further prevention of the remodeling process, salvage of viable myocardium, and improvement in cardiac function.

**Discussion**

**[0297]** This study demonstrates that ELR+ chemokines produced by ischemic tissues regulate the development of compensatory vasculogenesis at ischemic sites by producing a chemoattractant gradient for bone marrow-derived endothelial cell precursors. Although both the ELR+ CXC chemokine IL-8 and the ELR- CXC chemokine SDF-1 demonstrate similar effects on chemotaxis of CD34+ endothelial precursors, as well as on mature endothelium (73), when expressed at different extravascular sites they impart opposing biological effects on directional egress of endothelial progenitors, and consequently on tissue neovascularization. By understanding these interactions we were able to manipulate and augment the chemotactic properties of a specific subset of human bone marrow-derived CD34+ cells in order to increase myocardial trafficking, induce infarct bed vasculogenesis, reduce post-ischemic ventricular remodeling, and improve myocardial function.

**[0298]** Since migration of bone marrow-derived progenitors through basement membrane is dependent on secretion of proteolytic enzymes such as metalloproteinase-9 (MMP-9, Gelatinase B) (81), intracardiac metalloproteinase activity

may be a critical determinant of angioblast extravasation from the circulation and transendothelial migration into the infarct zone. IL-8 induces rapid release (within 20 minutes) of the latent form of MMP-9 from intracellular storage granules in neutrophils (82-83), and increases serum MMP-9 levels by up to 1,000-fold following intravenous administration in vivo in non-human primates (84). Since IL-8 significantly increases MMP-9 expression in bone marrow progenitors (81), and neutralizing antibodies against MCP-9 prevent mobilization of these cells (85), the results of our study suggest that angioblast infiltration and subsequent infarct bed vasculogenesis may result from IL-8-dependent increases in MMP-9 secretion.

[0299] Activation of latent MMP-9 and concomitant development of neoangiogenesis within murine myocardial infarct scar tissue has been shown to depend on urokinase-type plasminogen activator (u-PA) co-expressed by bone marrow progenitors infiltrating the infarct bed (81). Transcription and proteolytic activity of u-PA in human cells is significantly increased by G-CSF and other colony stimulating factors (86-88). Since IL-8-induced chemotaxis and progenitor mobilization require the presence of additional signals delivered through functional G-CSF receptors (89), it is possible that increased u-PA activity is required for IL-8 mediated trafficking of angioblasts to sites of ischemia. This would explain the limited extent of infarct bed neoangiogenesis observed normally after myocardial infarction (62, 63) despite high levels of IL-8 production, and provides a rationale for in vivo or ex vivo administration of colony stimulating factors to mobilize and differentiate human bone marrow-derived angioblasts for use in therapeutic revascularization of ischemic tissues.

[0300] Constitutive production of the CXC chemokine SDF-1 by bone marrow stromal cells appears to be essential for bone marrow homing and engraftment of haematopoietic progenitors (76-78). In addition, expression of SDF-1 in non-haematopoietic tissues plays a role in the developing vascular system since SDF-1<sup>-/-</sup> mice have defects in both vascularization of the gastrointestinal tract (50) and ventricular septum formation (90). Since bone marrow-derived endothelial precursors express CXCR4 (80) and demonstrate chemotactic responses to SDF-1, as shown here, induced expression of SDF-1 at non-haematopoietic sites during embryogenesis or following tissue injury may be an important element in the process of tissue neovascularization (91). Our ability to redirect trafficking of human bone marrow-derived angioblasts to sites of tissue ischemia by interruption of CXCR4/SDF-1 interactions argues strongly that SDF-1 is a biologically active chemotactic factor for human endothelial precursors, and that it may have pro-angiogenic activity if expressed at non-haematopoietic sites. Future studies will address whether increased expression and localization of SDF-1 and other chemokines at the sites of tissue ischemia might be synergistic with ELR+ CXC chemokines in augmenting vasculogenesis. Together, the results of this study indicate that CXC chemokines, including IL-8, Gro- $\alpha$ , and SDF-1, play a central role in regulating human bone marrow-dependent vasculogenesis, and that manipulation of interactions between these chemokines and their receptors on autologous human bone marrow-derived angioblasts can enhance the potential efficacy of therapeutic vasculogenesis following tissue ischemia.

### Third Series of Experiments

#### Experimental Procedures and Results

[0301] 1. Myocardial Ischemia Induces Production Of CC Chemokines and Increases Human CD34+ Angioblast Expression Of CC Chemokine Receptors

[0302] Since human mononuclear cell chemotaxis and tissue infiltration is regulated by interactions between cell surface receptors with specific chemokine ligands, the effects of ischemia on angioblast CC chemokine receptor expression and on kinetics of CC chemokine production by infarcted rat myocardium were investigated. As shown in FIG. 12, culture of CD34+CD117<sup>bright</sup> angioblasts with serum from LAD-ligated rats increased surface expression of CCR1 and CCR2, while surface expression of CCR3 and CCR5 remained unchanged.

[0303] As shown in FIG. 13, infarcted myocardium demonstrated a time-dependent increase in mRNA expression of several CCR-binding chemokines. Infarcted myocardium was found to express over 8-fold higher levels of the CCR2-binding CC chemokine MCP-1, and 3-3.5-fold higher mRNA levels of MCP-3 and RANTES, as well as the CCR3-binding chemokine eotaxin, after normalizing for total mRNA content (all  $p < 0.001$ ). This pattern of gene expression appeared to be relatively specific since every infarcted tissue studied demonstrated increased expression of these CC chemokines and none demonstrated induced expression of the CCR5-binding CC chemokines MIP-1  $\alpha$  or MIP-1  $\beta$ .

[0304] 2. Trafficking Of Human CD34+ Angioblasts to Ischemic Myocardium is Regulated by Induced Expression of CC and CXC Chemokines

[0305] Next investigated was whether human angioblast trafficking to ischemic myocardium was related to the induced expression of the CC chemokines identified above. Co-administration of blocking mAbs against MCP-1, MCP-3, and RANTES, or against eotaxin, reduced myocardial trafficking of human angioblasts by 40-60% relative to control antibodies ( $p < 0.01$ ), FIG. 14. To prove that CC chemokines mediate angioblast chemotaxis to ischemic myocardium, we measured in vivo angioblast chemotaxis in response to eotaxin. As shown in FIG. 15, intracardiac injection of eotaxin into non-infarcted hearts induced 1.5-1.7 fold increase in CD34+ angioblast trafficking whereas injection of the growth factors VEGF and stem cell factor had no effect on chemotaxis despite increasing angioblast proliferation (not shown).

[0306] Fourth Series of Experiments

[0307] Determination of Myocyte Size. Myocyte size was measured in normal rat hearts and in the infarct zone, peri-infarct rim and distal areas of infarct tissue sections stained by trichrome. The transverse and longitudinal diameters (mm) of 100-200 myocytes in each of 10-15 high-powered fields were measured at 400 $\times$  using Image-Pro Plus software.

[0308] Measurement of Myocyte Apoptosis by DNA End-Labeling of Paraffin Tissue Sections.

[0309] For in situ detection of apoptosis at the single cell level we used the TUNEL method of DNA end-labeling mediated by dextrynucleotidyl transferase (TdT) (Boehringer

Mannheim, Mannheim, Germany). Rat myocardial tissue sections were removed from LAD-ligated rats at two weeks after injection of either saline or CD34+ human cells, and from healthy rats as negative controls. Briefly, tissues were deparaffinized, digested with Proteinase K, and incubated with TdT and fluorescein-labeled dUTP in a humid atmosphere for 60 minutes at 37° C. After incubation for 30 minutes with an antibody specific for fluorescein conjugated alkaline phosphatase the TUNEL stain was visualized in which nuclei with DNA fragmentation stained blue.

[0310] 1. Neangiogenesis Protects Hypertrophied Myocardium Against Apoptosis.

[0311] The mechanism by which induction of neo-angiogenesis resulted in improved cardiac function was investigated. Results showed that two weeks after LAD ligation the myocytes in the peri-infarct rim of saline controls had distorted appearance, irregular shape, and similar diameter to myocytes from rats without infarction (0.020 mm  $\pm$  0.002 vs 0.019 mm  $\pm$  0.001). In contrast, the myocytes at the peri-infarct rim of rats who received CD34+ cells had regular, oval shape, and were significantly larger than myocytes from control rats (diameter 0.036 mm  $\pm$  0.004 vs 0.019 mm  $\pm$  0.001,  $p < 0.01$ ). By concomitant staining for the myocyte-specific marker desmin and DNA end-labeling, 6-fold lower numbers of apoptotic myocytes were detected in infarcted left ventricles of rats injected with CD34+ cells compared with saline controls (apoptotic index 1.2 $\pm$ 0.6 vs 7.1 $\pm$ 0.7,  $p < 0.01$ ). These differences were particularly evident within the peri-infarct rim, where the small, irregularly-shaped myocytes in the saline-treated controls had the highest index of apoptotic nuclei. In addition, whereas apoptotic myocytes extended throughout 75-80% of the left ventricular wall in saline controls, apoptotic myocytes were only detectable for up to 20-25% of the left ventricle distal to the infarct zone in rats injected with CD34+ cells. Together, these results indicate that the infarct zone vasculogenesis and peri-infarct angiogenesis induced by injection of CD34+ cells prevented an eccentrically-extending pro-apoptotic process evident in saline controls, enabling survival of hypertrophied myocytes within the peri-infarct zone and improving myocardial function.

[0312] 2. Early Neoangiogenesis Prevents Late Myocardial Remodeling.

[0313] The last series of experiments showed the degree of peri-infarct rim myocyte apoptosis at two weeks in control and experimental groups (saline vs CD34+ cells) compared with progressive myocardial remodeling over the ensuing four months. Despite similar initial reductions in LVEF and increases in LVAS, by two weeks the mean proportion of collagenous deposition or scar tissue/normal left ventricular myocardium, as defined by Masson's trichrome stain, was 3% in rats receiving CD34+ cells compared with 12% for those receiving saline. By 15 weeks post-infarction, the mean proportion of scar/normal left ventricular myocardium was 13% in rats receiving CD34+ cells compared with 36-45% for each of the other groups studied (saline, CD34-, SVEC) ( $p < 0.01$ ). Rats receiving CD34+ cells demonstrated significantly increased mass of viable myocardium within the anterior free wall which comprised myocytes exclusively of rat origin, expressing rat but not human MHC molecules, confirming intrinsic myocyte salvage rather than myocyte regeneration from human stem cell precursors. Whereas

collagen deposition and scar formation extended almost through the entire left ventricular wall thickness in controls, with aneurysmal dilatation and typical EKG abnormalities, the infarct scar extended only to 20-50% of the left ventricular wall thickness in rats receiving CD34+ cells. Moreover, pathological collagen deposition in the non-infarct zone was markedly reduced in rats receiving CD34+ cells. Together, these results indicate that the reduction in peri-infarct myocyte apoptosis observed at two weeks resulted in prolonged survival of hypertrophied, but viable, myocytes and prevented myocardial replacement with collagen and fibrous tissue by 15 weeks.

#### Discussion

[0314] The observation that proliferating capillaries at the peri-infarct rim and between myocytes were of rat origin shows that in addition to vasculogenesis human angioblasts or other co-administered bone-marrow derived elements may be a rich source of pro-angiogenic factors, enabling additional induction of angiogenesis from pre-existing vasculature.

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1. A method of stimulating vasculogenesis in ischemia-damaged tissue of a subject comprising:

- (a) removing stem cells from a location within the subject;
- (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and
- (c) introducing the endothelial progenitor cells from step (b) into a different location within the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.

2. The method of claim 1, wherein the endothelial progenitor cells are autologous.

3. The method of claim 1, wherein subsequent to step (b), but before step (c), the endothelial progenitor cells are expanded by contacting them with a growth factor.

4. The method of claim 3, wherein the growth factor is specific for, or primarily has effects upon endothelial cells.

5. The method of claim 3, wherein the growth factor is a cytokine.

6. The method of claim 5, wherein the cytokine is VEGF, PGF, G-CSF, IGF, M-CSF, or GM-CSF.

7. The method of claim 3, wherein the growth factor is a chemokine.

8. The method of claim 7, wherein the chemokine is Interleukin-8.

9. The method of claim 3, wherein the endothelial progenitor cells are separated from other stem cells before expansion.

10. The method of claim 1, wherein the ischemia-damaged tissue is myocardium.

11. The method of claim 1, wherein the subject has suffered a myocardial infarct.

12. The method of claim 1, wherein the ischemia-damaged tissue is nervous system tissue.

13. The method of claim 1, wherein the subject has suffered a cerebral ischemic event.

14. The method of claim 1, wherein the subject is deemed at risk from a cerebral ischemic event.

15. The method of claim 1, wherein the stem cells are removed from the subject's bone marrow.

16. The method of claim 15, wherein the removal of the stem cells from the bone marrow is effected by aspiration from the subject's bone marrow.

17. The method of claim 1, wherein the removal of the stem cells from the subject is effected by a method comprising:

- (a) introducing a growth factor into the subject to mobilize the stem cells into the subject's blood; and

(b) removing a sample of blood containing the stem cells from the subject.

18. The method of claim 17, wherein the growth factor is introduced into the subject subcutaneously, orally, intravenously or intramuscularly.

19. The method of claim 17, wherein the growth factor is a chemokine that induces mobilization.

20. The method of claim 19, wherein the chemokine is Interleukin-8.

21. The method of claim 17, wherein the growth factor is a cytokine.

22. The method of claim 21, wherein the cytokine is G-CSF, M-CSF, or GM-CSF.

23. The method of claim 1, wherein the endothelial progenitor cells are recovered based upon their expression of CD117.

24. The method of claim 1, wherein the endothelial progenitor cells are recovered based upon their expression of a GATA-2 activated gene product.

25. The method of claim 1, wherein the endothelial progenitor cells are recovered based upon their expression of one or more of CD34, VEGF-R, Tie-2, GATA-3 or AC133.

26. The method of claim 1, wherein the subject has suffered or is suffering from one or more of the following: myocardial infarction, chronic heart failure, ischemic heart disease, coronary artery disease, diabetic heart disease, hemorrhagic stroke, thrombotic stroke, embolic stroke, limb ischemia, or another disease in which tissue is rendered ischemic.

27. A method of treating acute myocardial infarct comprising the method of claim 1, wherein the subject is suffering acute myocardial infarct.

28. The method of claim 1, wherein step (a) occurs prior to the subject suffering ischemia-damaged tissue and wherein step (c) occurs after the subject has suffered ischemia-damaged tissue.

29. The method of claim 1, wherein the endothelial progenitor cells are frozen for a period of time between steps (b) and (c).

30. The method of claim 3, wherein the endothelial progenitor cells are frozen for a period of time after being expanded but before step (c) is performed.

31. The method of claim 1, wherein the endothelial progenitor cells are introduced into the subject by injection directly into the peripheral circulation, heart muscle, left ventricle, right ventricle, coronary artery, cerebro-spinal fluid, neural tissue, ischemic tissue, or post-ischemic tissue.

32. The method of claim 1, further comprising administering to the subject one or more of the following: an inhibitor of Plasminogen Activator Inhibitor, Angiotensin

Converting Enzyme Inhibitor or a beta blocker, wherein such administration occurs prior to, concomitant with, or following step (c).

33. A method of stimulating angiogenesis in peri-infarct tissue in a subject comprising:

- (a) removing stem cells from a location within the subject;
- (b) recovering endothelial progenitor cells from the stem cells removed in step (a);
- (c) expanding the endothelial progenitor cells recovered in step (b) by contacting the progenitor cells with a growth factor; and
- (d) introducing the expanded endothelial progenitor cells from step (c) into a different location in the subject such that the endothelial progenitor cells stimulate angiogenesis in peri-infarct tissue in the subject.

34. A method of selectively increasing the trafficking of endothelial progenitor cells to ischemia-damaged tissue in a subject comprising administering to the subject endothelial progenitor cells and a chemokine so as to thereby attract the endothelial progenitor cells to the ischemia-damaged tissue.

35. The method of claim 34, wherein the endothelial progenitor cells have been derived from the subject's bone marrow.

36. The method of claim 34, wherein the chemokine is a CXC chemokine.

37. The method of claim 36, wherein the CXC chemokine is Interleukin-8, Gro-Alpha, or Stromal-Derived Factor-1.

38. The method of claim 34, wherein the chemokine is a CC chemokine.

39. The method of claim 38, wherein the CC chemokine is RANTES, EOTAXIN, MCP-1, MCP-2, MCP-3, or MCP-4.

40. The method of claim 34, wherein the chemokine is administered to the subject by injection into the subject's peripheral circulation, heart muscle, left ventricle, right ventricle, a coronary artery, spinal fluid, neural tissue, ischemic tissue, or post-ischemic tissue.

41. The method of claim 34, wherein the endothelial progenitor cells are human cells.

42. The method of claim 34, wherein the ischemia-damaged tissue is myocardium.

43. The method of claim 34, wherein the ischemia-damaged tissue is neural tissue.

44. The method of claim 34, wherein the endothelial progenitor cells express CD117.

45. The method of claim 34, wherein the endothelial progenitor cells express at least one of CD34, GATA-2, GATA-3 or AC133.

46. The method of claim 34, wherein the chemokine is administered to the subject by injection into the subject's peripheral circulation, heart muscle, left ventricle, right ventricle, coronary arteries, cerebro-spinal fluid, neural tissue, ischemic tissue, or post-ischemic tissue.

47. The method of claim 1, 33 or 34, wherein the subject is mammalian.

48. The method of claim 47, wherein the subject is human.

49. A method of increasing trafficking of endothelial progenitor cells or angioblasts to ischemia-damaged tissue in a subject comprising inhibiting any interaction between Stromal-Derived Factor-1 and CXCR4.

50. The method of claim 49, wherein interaction between Stromal-Derived Factor-1 (SDF-1) and CXCR4 is inhibited

by administration of an anti-SDF-1 or an anti-CXCR4 monoclonal antibody to the subject.

51. The method of claim 50, further comprising administering to the subject an Angiotensin Converting Enzyme Inhibitor, an AT<sub>1</sub>-receptor blocker, or a beta blocker.

52. A method of reducing trafficking of endothelial progenitor cells to bone marrow in a subject comprising inhibiting production of Stromal-Derived Factor-1 in the subject's bone marrow.

53. The method of claim 52, wherein SDF-1 production is inhibited by administration of an anti-SDF-1 or anti-CXCR4 monoclonal antibody to the subject.

54. A method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific chemokine produced by proliferating cells associated with the cancer so as to reduce trafficking of endothelial progenitor cells to such proliferating cells and thereby treat the cancer in the subject.

55. A method for treating a cancer in a subject comprising administering to the subject a monoclonal antibody directed against an epitope of a specific receptor located on an endothelial progenitor cell, for a chemokine produced by proliferating cells associated with the cancer, so as to reduce trafficking of the endothelial progenitor cell to such proliferating cells and thereby treat the cancer in the subject.

56. The method of claim 55, wherein the receptor is CXCR1, CXCR2 or VEGF-R.

57. A method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce vasculogenesis in the subject's tumor and thereby treat the tumor.

58. A method for treating a tumor in a subject comprising administering to the subject an antagonist to a specific receptor on an endothelial progenitor cell so as to reduce the progenitor cell's ability to induce angiogenesis in the subject's tumor and thereby treat the tumor.

59. The method of claim 57 or 58, wherein the receptor is a CD117 receptor.

60. The method of claim 54, 55, 57, or 58, wherein the subject is mammalian.

61. The method of claim 60, wherein the subject is human.

62. A method for expressing a gene of interest in an endothelial progenitor cell or a mast progenitor cell which comprises inserting into the cell a vector comprising a promoter containing a GATA-2 motif and the gene of interest.

63. The method of claim 62, wherein the vector is inserted into the cell by transfection.

64. The method of claim 62 wherein the promoter is a preproendothelin-1 promoter.

65. The method of claim 64, wherein the promoter is of mammalian origin.

66. The method of claim 65, wherein the promoter is of human origin.

67. A composition comprising an amount of a monoclonal antibody directed against an epitope of a specific chemokine produced by a cancer effective to reduce trafficking of endothelial progenitor cells to the cancer, and a pharmaceutically acceptable carrier.



68. A method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

- (a) removing stem cells from a location within the subject;
- (b) recovering endothelial progenitor cells from the stem cells removed in step (a);
- (c) recovering those endothelial progenitor cells recovered in step (b) that express GATA-2;
- (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and
- (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality.

69. A method of treating an abnormality in a subject wherein the abnormality is treated by the expression of a GATA-2 activated gene product in the subject comprising:

- (a) removing stem cells from a location within the subject;
- (b) recovering mast progenitor cells from the stem cells removed in step (a);
- (c) recovering those mast progenitor cells recovered in step (b) that express GATA-2;
- (d) inducing the cells recovered in step (c) as expressing GATA-2 to express a GATA-2 activated gene product; and
- (e) introducing the cells expressing a GATA-2 activated gene product from step (d) into a different location in the subject such as to treat the abnormality.

70. The method of claims 68 or 69, wherein the abnormality is ischemia-damaged tissue.

71. The method of claims 68 or 69, wherein the gene product is proendothelin.

72. The method of claims 68 or 69, wherein the gene product is endothelin.

73. A method of improving myocardial function in a subject that has suffered a myocardial infarct comprising:

- (a) removing stem cells from a location in the subject;
- (b) recovering cells that express CD117 from the stem cells; and
- (c) introducing the recovered cells into a different location in the subject such that the cells improve myocardial function in the subject.

74. The method of any of claims 68, 69, or 73, wherein the subject is of mammalian origin.

75. The method of claim 74, wherein the mammal is of human origin.

76. A method of stimulating vasculogenesis in ischemia-damaged tissue in a subject comprising:

- (a) obtaining allogeneic stem cells;
- (b) recovering endothelial progenitor cells from the stem cells removed in step (a); and
- (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate vasculogenesis in the subject's ischemia-damaged tissue.

77. The method of claim 76, wherein the allogeneic stem cells are obtained from embryonic, fetal or cord blood sources.

78. A method of stimulating angiogenesis in ischemia-damaged tissue in a subject comprising:

- (a) obtaining allogeneic stem cells;
- (b) recovering endothelial progenitor cells in the stem cells removed in step (a); and
- (c) introducing the endothelial progenitor cells recovered in step (b) into the subject such that the endothelial progenitor cells stimulate angiogenesis in the subject's ischemia-damaged tissue.

79. The method of claim 78, wherein the allogeneic stem cells are obtained from embryonic, fetal or cord blood sources.

80. A method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting G-CSF into the subject in order to mobilize endothelial progenitor cells.

81. A method of improving myocardial function in a subject that has suffered a myocardial infarct comprising injecting anti-CXCR4 antibody into the subject.

82. The method of claim 81 further comprising introducing endothelial progenitor cells into the subject.

83. The method of claim 82 further comprising introducing G-CSF into the subject in order to mobilize endothelial progenitor cells.

84. An endothelial progenitor cell that expresses CD117.

85. An endothelial progenitor cell that expresses one or more of the group consisting of GATA-2, GATA-3, CD34, AC133, CD34 and CD117.

86. The endothelial progenitor cell of claim 85, wherein the progenitor cell is derived from bone marrow.

87. A method of expanding an endothelial progenitor cell population comprising contacting an endothelial progenitor cell with a cytokine.

88. The method of claim 87, wherein the cytokine is selected from the group consisting of G-CSF, GM-CSF, M-CSF, VEGF, and FGF.

89. A method of expanding an endothelial progenitor cell population comprising contacting an endothelial progenitor cell with a chemokine.

90. The method of claim 89, wherein the chemokine is a CC chemokine.

91. The method of claim 90, wherein the CC chemokine is selected from the group consisting of RANTES, EOTAXIN, MCP-1, MCP-2, MCP-3, and MCP-4.

92. A method of identifying bone marrow-derived endothelial progenitor cells comprising recovering progenitor cells based on their expression of CD117.

93. A method of identifying bone marrow-derived endothelial progenitor cells comprising recovering progenitor cells based on their expression of any or all of the group consisting of GATA-2, GATA-3, CD34, AC133, CD34 and CD117.

94. The method of claim 1, wherein the removal of the stem cells from the subject is effected by a method comprising:

- (a) eliciting production of growth factor in the subject to mobilize the stem cells into the subject's blood; and
- (b) removing a sample of blood containing the stem cells from the subject.

95. The method of claim 94, wherein the growth factor production is elicited by a gene therapy technique.

96. A method of selectively increasing the trafficking of endothelial progenitor cells to ischemia-damaged tissue in a subject comprising eliciting chemokine production in the subject so as to thereby attract the endothelial progenitor cells to the ischemia-damaged tissue.

97. The method of claim 96, wherein the chemokine production is elicited by a gene therapy technique.

98. The method of claim 97, further comprising administering endothelial progenitor cells to the subject.

\* \* \* \* \*